In-bead screening

Nielsen, Thomas Eiland; Qvortrup, Katrine

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(71) Applicant: **DANMARKS TEKNISKE UNIVERSITET** [DK/DK]; Anker Engelundsvej 1, Bygning 101A, 2. sal, DK-2800 Kgs. Lyngby (DK).

(72) Inventors: **NIELSEN, Thomas, E.**; Ordrupvej 55B, DK-2920 Charlottenlund (DK). **QVORTRUP, Katrine**; Södra Förstadsgatan 121G, S-21428 Malmö (SE).

(74) Agent: **OLSEN, Lars Pallisgaard**; Guardian IP Consulting I/S, Diplomvej, Building 381, DK-2800 Kgs. Lyngby (DK).

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(54) **Title**: IN-BEAD SCREENING

(57) **Abstract**: The present invention relates to screening of one-bead-one-compound (OBOC) combinatorial libraries which is useful for the discovery of compounds displaying molecular interactions with a biological or a physicochemical system, such as substrates and inhibitors of enzymes and the like. The invention provides a method for screening a library of compounds for their interaction with a physicochemical or biological system and a corresponding kit for performing the method of screening a one-bead-one-compound library of compounds.

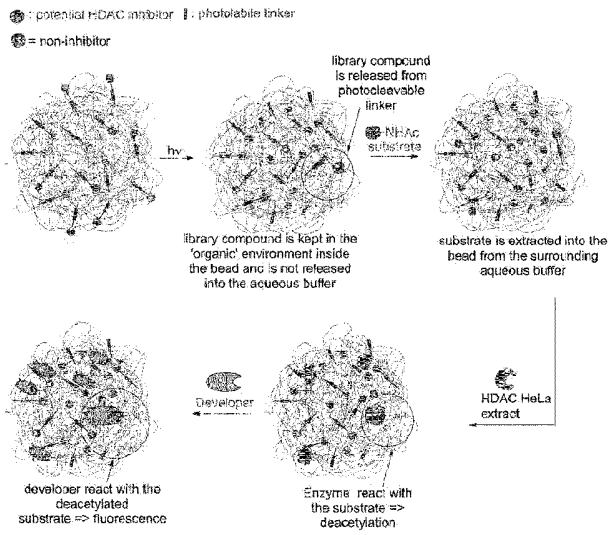

Figure 5

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IN-BEAD SCREENING

BACKGROUND OF THE INVENTION

Field of the invention

The present invention relates to screening of one-bead-one-compound (OBOC) combinatorial libraries which is useful for the discovery of compounds displaying molecular interactions with a biological or a physicochemical system, such as substrates and inhibitors of enzymes and the like.

Prior art

The screening of one-bead-one-compound (OBOC) combinatorial libraries is useful for the discovery of compounds displaying molecular interactions with a biological or a physicochemical system, such as substrates and inhibitors of enzymes and the like. The method is particularly attractive since hundreds of thousands of chemical compounds can be generated via split-pool synthesis and screened in a short time. In addition, each compound is localized on an individual bead and therefore spatially addressable during hit identification. The basic idea of screening ligands directly on bead was recognized by Lam et al. as early as 1991 (K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82–84.). Many steps have since been taken to realize this concept and reported progress range from optimization of screening conditions ((a) H.-S. Lim, C. T. Archer, T. Kodadek, *J. Am. Chem. Soc.* **2007**, *129*, 7750-7751; (b) M. G. Paulick, K. M. Hart, K. M. Brinner, M. Tjandra, D. H. Charychand, R. N. Zuckermann, *J. Comb. Chem.* **2006**, *8*, 417–426; (c) T. Kodadek, K. Bachhawat-Sikder, *Mol. Biosyst.* **2006**, *2*, 25–35; (d) A. Lehman, S. Gholami, M. Hahn, K. S. Lam, *J. Comb. Chem.* **2006**, *8*, 562–570; (e) A. Song, J. Zhang, C. B. Lebrilla, K. S. Lam, *J. Am. Chem. Soc.* **2003**, *125*, 6180–6188; (f) M. Meldal, *Biopolymers* **2002**, *66*, 93–100), to the development of suitable library decoding techniques ((a) E. M. V. Johansson, J. Dubois, T. Darbre, J.-L. Reymond, *Bioorg. Med. Chem.* **2010**, *18*, 6589–6597; (b) S. André, C. E. P. Maljaars, K. M. Halkes, H.-J. Gabius, J. P. Kamerling, *Bioorg. Med. Chem. Lett.* **2007** ,*17*, 793–798. (c) M. Kubo, R. Nishimoto, M. Doi, M. Kodama, H.

Hioki, *Chem. Commun.* **2006**, 3390–3392; (d) R. S. Youngquist, G. R. Fuentes, M. P. Lacey, T. Keough, *Rapid Commun. Mass Spectrom.* **1994**, *8*, 77-81).

A drawback of on-bead assays is the inherent attachment of the compound to the solid support, which may not display the same activity as that of the free molecule in solution ((a) M. M. Marani, M. C. M. Ceron, S. L. Giudicessi, E. de Oliveira, S. Coté, R. Erra-Balsells, F. Albericio, O. Cascone, S. A. Camperi, *J. Comb. Chem.* **2009**, *11*, 146–150; (b) S. Dixon, S., K.T. Ziebart, Z. He, M. Jeddeloh, C. L. Yoo, X. Wang, A. Lehman, K. S. Lam, M. D. Toney, M. J. Kurth, *J. Med. Chem.* **2006**, *49*, 7413–7426; (c) L. I. Robins, S. M. Dixon, D. K. Wilson, M. J. Kurth, *Biorg. Med. Chem.* **2006**, *14*, 7728-7735. (d) S. Dixon, L. Robins, R. A. Elling, R. Liu, K. S. Lam, D. K. Wilson, M. J. Kurth, Bioorg. *Med. Chem. Lett.* **2005**, *15*, 2938–2942.). For example, immobilized molecules may exhibit unfavorable kinetics in binding to the target protein due to the interference by the linker or solid support, which may lead to false negatives. Commercial resins typically used for library synthesis often have a high loading, which is necessary to provide sufficient amounts of material for subsequent hit identification. However, a high ligand density may also result in unwanted ligand interaction and/or nonspecific binding (X. Chen, P. H. Tan, Y. Zhang, D. Pei, *J. Comb. Chem.* **2009**, *11*, 604–611). A high ligand density makes it possible for a target molecule to bind to beads displaying moderate- or even low-affinity ligands. Therefore, the true potency of the identified ligands is only revealed after time-consuming and expensive large scale re-synthesis and testing in solution.

To establish a direct link between on-bead library synthesis and solution phase screening, research efforts both in academia and industry have focused on the development of suitable off-bead screening strategies. Such strategies include the use of gels to embed the beads in a matrix that reduce free diffusion of the library members when released from the beads ((a) R. F. Harris, A. J. Nation, G. T. Copeland, S. J. Miller, *J. Am. Chem. Soc.* **2000**, *122*, 11270-11271; (b) M. Müller, T. W. Mathers, A. P. Davis, *Angew. Chem., Int. Ed.* **2001**, *40*, 3813-3815; (c) K. J. Johansson, M. R. M Andrea, A. Berkessel, A. P. Davis, *Tetrahedron Lett.* **2005**, *46*, 3923-3926; (d) N. Maillard, T. Darbre J. L. Reymond, *J. Comb. Chem.* **2009**, *11*, 667-675.), or the use of microarrays in which the beads are separated from each other in individual wells ((a) Grégory Upert, Christoph A. Merten, Helma Wen-

nemers, *Chem. Commun*. **2010**, *46*, 2209–2211 (b) J. R. Schullek, J. H. Butler, Z.-J. Ni, D. Chen, Z. Yuan, *Anal. Biochem*. **1997**, *246*, 20-29; (c) A. J. You, R. J. Jackman, G. M. Whitesides, S. L. Schreiber, *Chem. Biol*. **1997**, *4*, 969-975; (d) A. Borchardt, S. D. Liberles, S. R. Biggar, G. R. Crabtree, S. L. Schreiber, *Chem. Biol*. **1997**, *4*, 961-968; (e) L. A. Walling, N. R. Peters, E. J. Horn, R. W. King, *J. Cell. Biochem*. **2001**, *84*, 7-12). The use of gels is limited by the fact that the assay environment is determined by the nature of the gel. Furthermore, post-screen bead isolation and hit identification is not straightforward. Off-bead screens using single bead nanowells also have several drawbacks. The creation of small molecule microarrays requires significant robotics capabilities to segregate beads into the wells and process them in an automated fashion. In addition, the use of microarrays are complicated by several factors including inhomogeneous filling of nanowells with beads and substrates, a low density of nanowells per area and difficulties to sequentially fill the compartments with beads and substrates.

Thus, there is a need in the technical field to circumvent some of the substantial challenges associated with on-bead and off-bead screening.

SUMMARY OF THE INVENTION

The present invention concerns biological screening assays that have been developed to allow the evaluation of covalently detached one-bead-one-compound (OBOC) combinatorial libraries of compounds for their ability to interact with a physicochemical or a biological system inside the organic "micro-compartments" of solid supports, for example inside polymeric beads. High purity of released material permits the direct release of compounds into aqueous environments and serves to enhance throughput-screening techniques, which rely on release of molecules directly into the assay medium in the micro-compartment provided by the solid support.

In one aspect of the present invention, compounds covalently detached from the solid supports, e.g. polymeric beads by photolysis, remain inside the solid supports/beads, when they are swelled in aqueous buffers. Such compounds readily

leave the solid support/beads upon washing with organic solvents. A measure of this phenomenon is given by the partition coefficient between the aqueous buffer and the organic environment inside the solid support/bead. Thus, in aqueous buffer each solid support/bead comprises a spatially separated 'micro-compartment' into which a compound can be released, or taken in from the surrounding media. In this way biological and other assays can be carried out inside the solid supports/beads as part of a method for screening a library of compounds for their interaction with a physicochemical or biological system, comprising:

a) obtaining a one-bead-one-compound library wherein the compounds are immobilized in solid supports,

b) covalently detaching the library compounds from the solid supports, so that each compound physically is located inside its parent solid support.

c) adding a physicochemical or biological system to said compound library in an aqueous media,

d) detecting solid support(s) in said library showing interaction between said compound(s) and the physicochemical or biological system, and

e) identifying compound(s) immobilized in said solid support(s) showing said interaction.

In a specific embodiment, immobilized compounds are released inside solid supports/beads by photolytic, chemical or enzymatic cleavage in an aqueous media before the physicochemical or biological system is added.

In another embodiment, the physicochemical or biological system is added to the solid supports/beads in an aqueous media before the immobilized compounds are released inside the beads.

Screening a library of compounds for their interaction with a physicochemical or biological system, where release of the immobilized compounds is required for the interaction, further requires that the compounds in the one-bead-one-compound library are immobilized on the solid support through a photolytically and/or chemically and/or enzymatically cleavable linker. In order to run the assay, the immobilized compounds are released inside the beads by either a photolytic, chemical or

enzymatic cleavage in an appropriate polar solvent, such as an aqueous buffer. In a preferred embodiment, the linker is a photolabile or enzymatic linker, allowing acids and/or bases to be used during construction of the library.

Compounds that may be screened according to the present inventions comprise non-oligomeric compounds, i.e. small molecules, and oligomeric compounds, i.e. oligo- and polypeptides, peptidomimetics, oligo- and polysaccharides, oligo- and polynucleotides. Such compound(s) could be a potential inhibitor, an agonist, an antagonist, a transcription factor, a substrate for an enzyme, a catalyst, a toxin, a hormone, an antigen, an antibody or a fragment thereof, a polynucleotide, or a derivative thereof.

The physicochemical or biological system may be selected from the group consisting of proteins, such as an enzyme, receptor, toxin, hormone, antibody or fragment thereof, antigen, polynucleotide, such as a gene or a regulator region, i.e. strands of DNA, or a genetic transcript, i.e. strands of RNA.

The present invention also concerns a kit for screening a one-bead-one-compound library of compounds comprising:

a) a plurality of solid supports, each solid support having immobilized therein a plurality of compounds in a "one-bead-one-compound" fashion, the compounds being immobilized on the solid support optionally through a photolytically and/or chemically and/or enzymatically cleavable linker,

b) one or more aqueous media(s) for washing and/or containing the solid supports,

c) a physicochemical or biological system for interaction with said compounds, and

d) a substrate for detecting an interaction between said compounds and the physicochemical or biological system.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Experimental setup used in photolysis reactions.

Figure 2: Illustration of the distribution of organic compounds inside polymeric beads in aqueous and organic media, respectively.

Figure 3: Illustration of a post-screening structure determination strategy.

Figure 4: Illustration of the "split-and-mix" synthesis.

Figure 5: Illustration of the "in-bead" screening technology for the identification of putative HDAC inhibitors, where the library molecule is a non-inhibitor.

Figure 6: Illustration of the "in-bead" screening technology for the identification of putative HDAC inhibitors, where the library molecule is an inhibitor.

Figure 7: Schematic illustration of experiments investigating control of substrate and inhibitor concentration.

Figure 8: Shows relative S/I ratio as a function of substrate concentration, and demonstrates that it is possible to control the concentration of substrate in the assay by variation of the concentration of the substrate solution.

Figure 9: Shows substrate HPLC area as a function of substrate concentration, and demonstrates that at low substrate concentrations (>100μM) all substrate is extracted into the beads.

Figure 10: Shows relative S/I ratio as a function of photolysis time, and demonstrates that it is possible to control the concentration of inhibitor in the assay by variation of the photolysis time and/or the amperage of the light.

Figure 11: SAHA-containing beads (9c) showing HDAC-inhibitor activity when subjected to the "in-bead" HDAC-inhibitor assay. When the photolysis step was omitted no SAHA was released and no quenching of HDAC-activity was observed. Beads functionalized with a ligand devoid of HDAC-inhibitory activity (9d) resulted in no quenching of fluorescence following photolysis.

Figure 12: Representative microscopy images of small, bead-based library (9a-h) subjected to in-bead HDAC-inhibitor assay with a photolysis time of 2 min.

Figure 13: Representative microscopy images of small, bead-based library (9a-h) subjected to in-bead HDAC-inhibitor assay with a photolysis time of 0.5 min.

Figure 14: Schematic illustration of experiments investigating the possibility of performing in-bead dose-response assays.

Figure 15: Shows dose-response measurements for two HDAC inhibitors.

DETAILED DESCRIPTION OF THE INVENTION

It was surprisingly observed that organic compounds preferably stay localized in the organic environment inside a polymeric bead whenever the surrounding media constitutes an aqueous buffer. With a series of organic compounds, we experimented with the distribution of compounds inside polymeric beads in aqueous and organic media, respectively (figure 1). When an aqueous solution of an organic compound is added to polymeric beads, the organic compound is extracted into the organic environment inside the bead. However, the organic compound readily leaves the beads upon washing with an organic solvent. Furthermore, when photolabile beads functionalized with an organic compound are embedded in an aqueous buffer before photolysis, the compounds subsequently released by photolysis stay inside the bead and are not released into the surrounding buffer.

Definitions

'Covalently detached' compounds indicate that the covalent bond between the linker and the compound has been cleaved.

The term "solid support" means any insoluble polymeric material, limited primarily by capacity for swelling, light permeability and the capacity for derivatization to attach any of a number of chemically reactive groups as well as compatibility with the synthetic chemistry used for linker attachment and/or synthesis. Suitable support materials typically will be composed of material commonly used in peptide and polymer synthesis. To improve swelling properties quite porous beads, resins, or other supports work well and are often preferable. Particularly preferred materials include polystyrene.

Solid supports may be of any shape or size, such as roughly spherical. The supports need not necessarily be homogenous in size, shape or composition; although the supports usually and preferably will be uniform.

The terms 'photolysis', photolytic cleavage and similar terms mean irradiation with light at any wavelength that causes cleavage of a covalent chemical bond.

The term "chemical cleavage" means a reaction with any chemical reagent that causes cleavage of a covalent chemical bond, including acid- or base-mediated cleavage of carboxamides or ester (e.g. using TFA, or sodium hydroxide), and thiol-mediated cleavage of a disulfide bonds.

The term "enzymatic cleavage" means any enzyme-mediated cleavage of a covalent chemical bond and include chymotrypsin-catalyzed hydrolysis of an ester bond and the trypsin-mediated cleavage of a Glu-Lys bond (for reviews on enzyme cleavable linkers in solid phase synthesis, see: (a) R. Reents et al., Drug. Discovery Today **2002**, *7*, 71; (b) R. Reents et al., Adv. Synth. Catal. **2001**, *343*, 501).

An aqueous media or buffer is an aqueous solution consisting of a mixture of a weak acid and its conjugate base or a weak base and its conjugate acid. It has the property that the pH of the solution changes very little when a small amount of strong acid or base is added to it. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications. 'Buffers' comprise HEPES, PBS, etc., known to people skilled in the art.

The term "physicochemical or biological system": an integrated structure of components and subsystems capable of performing, in aggregate, one or more specific functions. A chemical or biological system may include, but are not limited to: binding of a ligand for a receptor of interest (e.g. GPCR), inhibition of an enzyme (e.g. HDAC), disruption of a protein/protein interaction (e.g. DNA replication), catalysis of a chemical transformation.

The term "identification" means any method to identify the compound synthesized on a bead of interest in a combinatorial library. Such methods include but are not limited to: (1) analysis of the released compound by mass spectrometry; (2) chemical tagging: reacting an individual marker with the solid support whenever a building block is coupled, thereby tracking the chemical history of each bead. Final

cleavage and analysis of the tagging molecules reveal a code for the structural identity of the compound formed. As an example, Ohlmeyer et al. in 1993 introduced a technique using a defined set of haloaromatic carbenes (Proc. Natl. Acad. Sci. USA 1993, 90, 10922-10926), (3) optical encoding: barcoded solid supports are used in the library synthesis. The code is read whenever a building block is coupled, thereby tracking the chemical history of each bead. As an example, Meldal et al. in 2010 introduced microparticle matrix encoding of beads (Angew. Chem. Int. Ed. Engl. 2010, 49, 3473-3476).

The present invention concerns in one aspect a method for screening a library of compounds for their interaction with a physicochemical or biological system, comprising:

a) obtaining a one-bead-one-compound library wherein the compounds are immobilized on a solid support,

b) covalently detaching the library compounds from the solid supports, so that each compound is physically located inside its parent solid support,

c) adding a physicochemical or biological system to said library in an aqueous media,

d) detecting bead(s) in said library showing interaction between said compound(s) and the physicochemical or biological system, and

e) identifying compound(s) immobilized in said bead(s) showing said interaction.

In a specific embodiment, immobilized compounds are releasing inside the solid support/beads by photolytic, chemical or enzymatic cleavage in an aqueous media before the physicochemical or biological system is added.

In another embodiment, the physicochemical or biological system is added to the beads in an aqueous media before the immobilized compounds are released inside the solid support/beads.

In one embodiment of the present invention, the method for screening a library of compounds involves the release of the compounds to facilitate an interaction with the physicochemical or biological system, and thus comprises the following steps:

a) obtaining a one-bead-one-compound library wherein the compounds are immobilized on a solid support through a photolytically and/or chemically and/or enzymatically cleavable linker,

b) releasing said immobilized compounds inside said beads by photolytic, chemical or enzymatic cleavage in an aqueous media,

c) adding a physicochemical or biological system to said library in an aqueous media,

d) detecting solid support(s)/bead(s) in said library showing interaction between said compound(s) and the physicochemical or biological system, and

e) identifying compound(s) immobilized in said solid support(s)/bead(s) showing said interaction.

The method may also include, or be followed by, assaying compounds of interest, such as identified positive compounds (hits), in a subsequent assay in order to verify the interaction(s), or to identify another interaction.

In a specific embodiment, immobilized compounds are released inside the beads by photolytic, chemical or enzymatic cleavage in an aqueous media before the physicochemical or biological system is added.

In another embodiment, the physicochemical or biological system is added to the solid supports/beads in an aqueous media before the immobilized compounds are released inside the beads.

In-bead screening provides an analytical method useful for determining the molecular interaction of a biological macromolecule, such as protein or a polynucleic acid, with a library compound, such as an agonist, antagonist, inhibitor, catalyst, reagent, or substrate inside the bead.

Said method is useful for identifying structure activity relationships of enzymes/substrates and enzymes/inhibitors. Enzymes comprise, but are not limited to

acylases, proteases, esterases, aldolases, oxidases, decarboxylases, kinases, carbonic anhydrases, lipases, phospholipases, phosphatases, cellulases, galactosidase, glycosyltransferases, exonucleases, polymerases, RNases, apyrases, heparinases, hyaluronidase, papain, ficin, elasatase, subtilisin and pronase.

Said method, useful for the determination of a biological interaction, comprises one or more components, such as a substrate structure, that allow the molecular interaction to be translated into an observable signal from the solids support/bead, and/or a chemical sensor that allows said molecular interaction to be translated into an observable signal from the bead.

The observable signal that links the biological interaction, such as the enzymatic conversion of a substrate into a product, can be of many kinds, such as a change in electrochemical properties, chromatographical properties, temperature, or preferably in spectral properties.

Observable spectral changes may directly result from structural differences between substrate and product, or indirectly, for example through a chemical indicator system, by processing the reaction product by secondary enzymes or reagents.

Particularly preferred observable spectral changes are those that induce changes in light absorbency or fluorescence in the bead. Recordable changes may rely on colorimetric or fluorimetric chemosensing of the molecular interaction under investigation, such as non-covalent binding interactions, covalent modifications, product formation, and substrate consumption. For example, a synthetic enzymatic substrate can be designed so that the enzyme turns a non-fluorescent or colorless appendage of the substrate into a fluorescent or colored product.

In one embodiment said enzymatic substrate, typically a synthetic or unnatural substrate mimicking the natural substrate, may contain cleavable esters or acetals of electron-deficient conjugated phenols, which upon cleavage liberates colored or fluorescent conjugate bases of said phenols. Said phenols include among other phenols known to persons skilled in the art para-nitrophenol (yellow), umbelliferone (blue fluorescent), resorufin (red fluorescent), and fluorescein (green fluorescent). Said phenyl esters can for example be substrates for lipases, esterases, or phospholipases, and said acetals can be substrates for pyranosidases.

In another embodiment said enzymatic substrate, typically a synthetic or unnatural substrate mimicking the natural substrate, may contain cleavable amides of electron-deficient anilines, also called anilides, which upon cleavage liberates the parent colored or fluorescent anilines. Said anilines include, among other anilines known to persons skilled in the art, para-nitroaniline (yellow). Said anilides can for example be substrates for trypsin, or chymotrypsin.

For substrates lacking a chromogenic or fluorogenic moiety, the product of the reaction may subsequently be converted into a chromogenic or fluorogenic moiety, often referred to as a coupled assay, either by another enzymatic assay, or by a chemical reagent. An enzymatically coupled assay could be the conversion of acetate, the product of a primary enzymatic transformation, into citrate accompanied by the formation of fluorescent NADH. A chemically coupled assay could be the conversion of naphthol, the product of the primary enzymatic transformation, into a colored azo dye by reaction with a diazonium salt.

Hydrolytic enzymatic reactions, such as the hydrolysis of esters and amides that liberate carboxylic acid products may be monitored by way of pH changes using buffer/indicator pairs with similar pKa values.

According to the present method, the physicochemical or biological system may include a substrate involved in detecting an interaction between the compound and the system inside the bead when added. Another advantageous aspect of the hydrophobic 'micro-compartment' inside the solid supports/beads is that such a substrate may be added to the solid supports/beads in an appropriate solvent and absorbed into the solid supports/beads in a step before or after the addition of the physicochemical or biological system.

Accordingly, the addition of the physicochemical or biological system and appropriate substrate may therefore have the following order:

i. adding a substrate for the physicochemical or biological system,

ii. allowing said substrate to be absorbed into said solid supports/beads,

iii. washing the solid supports/beads in an aqueous media,

iv. adding a physicochemical or biological system,

v. allowing said physicochemical or biological system to be absorbed into said solid supports/beads, and

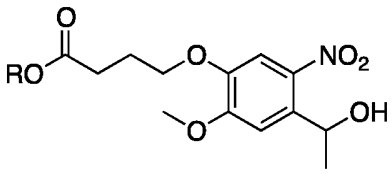
vi. washing the solid supports/beads in an aqueous media.

Alternatively, the addition of the physicochemical or biological system, including washing step(s) iv-vi may come before the addition of the substrate, including washing step(s) i-iii.

The physicochemical or biological system may be selected from the group consisting of proteins, such as an enzyme, receptor, toxin, hormone, antibody or fragment thereof, antigen, polynucleotide, such as a gene or a regulator region, i.e. strands of DNA, or a genetic transcript, i.e. strands of RNA.

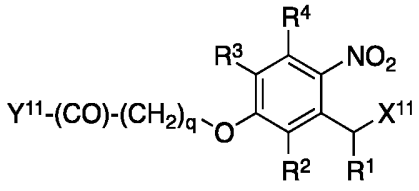
In the case of utilizing cleavable linkers in the library, cleavage of the immobilized compounds may be performed before addition of the substrate and the physicochemical or biological system, between addition of the substrate and the physicochemical or biological system, or after the substrate and the physicochemical or biological system have been added.

In accordance with the present invention, the compounds may be immobilized through a photolabile linker, i.e. a linker which is photolytically cleavable. A large number of photolabile linkers are known in the art (Scott: Linker Strategies in Solid-Phase Organic Synthesis 2009, John Wiley and Sons). Any such linker may be chosen if suited for the construction of the library. As non-limiting examples of such photolabile linkers can be mentioned linkers based on the o-nitroveratryl group independently introduced in the mid 1990s by Greenberg [J. Org. Chem. 1995, 60, 3358-3364] and Holmes [J. Org. Chem. 1995, 60, 2318-2319], for the solid-phase synthesis of oligonucleotides and peptides, respectively.
Photolabile linkers have been made to release a variety of functionalized molecules from solid supports, such as carboxamides, sulphonamides, carboxylic acids, alcohols, amines. For an overview on photolabile linkers for solid-phase organic synthesis, see: Scott: Linker Strategies in Solid-Phase Organic Synthesis 2009, John Wiley and Sons. As examples can be mentioned:
(1) Whitehouse et al, Tetrahedron Letters, 1997, 38, 7851-7852 discloses a photolabile linker for solid-phase organic synthesis having the structure:

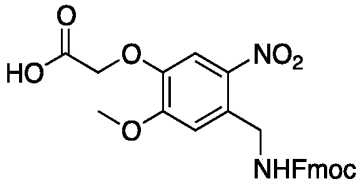


where R is hydrogen for use in solid-phase synthesis of carboxylic acids.

(2) WO 96/00378 discloses photolabile linkers for use in solid-phase synthesis, for example in the synthesis of small molecule and peptide libraries. The photolabile linking group is represented by the formula:

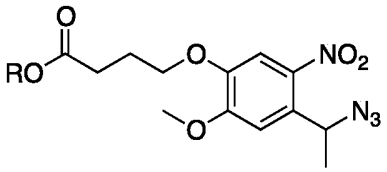


for example:



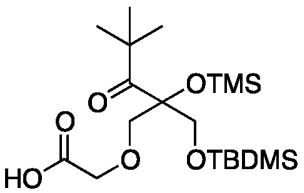
for use in solid-phase synthesis of carboxamides.

(3) Qvortrup and Nielsen, Chem. Commun. 2011, 47, 3278-3280 disclose an azido-functionalized photolabile linker having the following structure:



where R is hydrogen or ethyl for use in solid-phase synthesis of 4-substituted NH-1,2,3-triazoles.

(4) A pivaloyl-based photolabile linker was developed by Giese (Peukert, S.; Giese, B., J. Org. Chem. 1998, 63 (24)) for the synthesis of peptides:



Alternatively, the compounds may be immobilized through a chemically cleavable linker, i.e. the linker is cleavable by addition of a chemical reagent. Such linkers are known in the art (for an overview on chemically cleavable linkers for solid-phase organic synthesis, see: Scott: Linker Strategies in Solid-Phase Organic Synthesis 2009, John Wiley and Sons.) and may be chosen if suited for the construction of the library. As non-limiting examples of such linkers can be mentioned base-cleavable linkers (e.g. HMBA-linker cleaved by aqueous sodium hydroxide solution), disulfide linker system subject to disulfide exchange (e.g. W. Tegge, W. Bautsch, R. Frank, J. Pept. Sci. **2007**, 13, 693-699) and diazobenzene linker system cleaved under mild reducing conditions using sodium dithionite (S. H. L. Verhelst, M. Fonovíc, M. Bogyo, Angew. Chem. Int. Ed. **2007**, *46*, 1 – 4).

The compounds may also be immobilized through an enzymatically cleavable linker. Such linkers are known in the art (for an overview on enzymatically linkers for solid-phase organic synthesis, see: Scott: Linker Strategies in Solid-Phase Organic Synthesis 2009, John Wiley and Sons). Any such linker may be chosen if suited for the construction of the library. As non-limiting examples of such linkers can be mentioned trypsin-mediated cleavage of a Glu-Lys bond (J. Weiler, H. Gausepohl, N. Hauser, *et al.*; Nucleic Acids Res. **1997**, 25, 2792), and the phosphodiesterase-mediated cleavage of phosphodiester containing linker system (D. T. Elmore, D. J. S. Guthrie, A. D. Wallace, S. R. E. Bates, J. Chem. Soc. Chem. Commun. **1992**, 1033).

In a specific embodiment, the compounds are immobilized through a combination of two, or even more linkers, such as two different photolabile linkers (cleaved at different wavelengths), a chemically cleavable linker combined with a photolytically cleavable linker, a chemically cleavable linker combined with an enzymatically cleavable linker, a photolytically cleavable linker combined with an enzymatically cleavable linker, etc. By combining two differently cleavable linkers, a part of the immobilized compounds may be released in the assay involving a physicochemical or biological system by cleaving one linker, e.g. a photolabile linker, and another part of the remaining immobilized compounds is released by cleaving another linker, e.g. another photolabile linker (another wavelength of the light) or a chemically

cleavable linker, such as a base- or acid-labile linker, or an enzymatically cleavable linker for identification of the compound of the solid support/bead. Alternatively, a sequential release of the immobilized compounds may be achieved by using one photolabile linker and limiting the time during which the solid supports/beads are exposed to the light before the assay, such that only a fraction of the compounds are released inside the solid supports/beads before the assay. Exposure of the solid supports/beads of interest, such as those containing hit compounds, to more light releases material sufficient for the identification of the compound structure. Similarly, the degree of cleavage of chemically or enzymatically cleavable linkers may be controlled by neutralization or inhibition, as desired.

If desired, more sequential releases may be achieved for performing more assays on the library or selected, e.g. positive solid supports/beads. This could be advantageous in order to e.g. verify the biological efficiency of the compound in a positive solid support/bead, or test compounds in positive solid supports/beads for different properties.

In the method, the identification involves isolating positive solid support(s)/bead(s) showing interaction in the physicochemical and/or biological system, followed by structure elucidation of the compound(s) in the isolated solid support(s)/bead(s).

Beads of interest identified in an assay may be manually picked out using a micropipette. Beads can also be automatically sorted according to their fluorescent intensity using for example the COPAS^{TM} sorting instrument (C. Christensena,T. Groth, C. B. Schiødt, N. T. Foged, Morten Meldal, QSAR Comb. Sci. **2003**, 22, 737-744), which is commercially available from Union Biometica.

The identification may involve release of the immobilized compounds from said isolated solid support(s)/bead(s) by chemical or photolytic cleavage before the structure elucidation.

The identification of compound structures may result from: (1) analysis of released compound by mass spectrometry (e.g. MALDI-MS); (2) chemical tagging, where an individual marker reacts with the solid support whenever a building block is coupled, thereby tracking the chemical history of each bead. Final cleavage and analysis of the tagging molecules reveal a code for the structural identity of the com-

pound formed. As an example, Ohlmeyer et al. in 1993 introduced a technique using a defined set of haloaromatic carbenes (Proc. Natl. Acad. Sci. USA **1993**, 90, 10922-10926); (3) optical encoding, such as barcoded solid supports are used in the library synthesis. By reading the codes whenever a building block is coupled, the chemical history of each bead can be tracked. Optical encoding has the advantage that the last release and sequencing step can be omitted. As an example, Meldal et al. has introduced microparticle matrix encoded beads (Angew. Chem. Int. Ed. **2010**, 49, 3473-3476).

To facilitate post-screening MALDI-TOF MS sequencing of beads of interest, a 4-bromophenylalanine spacer was positioned between a Rink-linker functionalized support and the photolabile linker unit (figure 1). Orthogonal cleavage of the acid-labile Rink linker provides a cleavage product with sufficiently high mass to be out of range of low-mass noise and matrix ions typically seen during the MALDI-TOF MS analysis. Furthermore, the 4-bromophenylalanine spacer generates mass peaks with a characteristic bromine isotope pattern, so that the relevant peaks of the library products are readily identified by the presence of two peaks of equal intensity $[M+Na]^+$ (for the ^{79}Br-capped fragments) and $[M+2+Na]^+$ (for the corresponding ^{81}Br-capped fragments). The beads of interest are washed with aqueous buffer and CH_3CN to remove assay components before manually transferring one bead to a MALDI target. The bead of interest is swollen in TFA/CH_2Cl_2 (2 µL) on the MALDI target before being subjected to MALDI-TOF MS analysis.

Compounds that may be screened according to the present inventions comprise non-oligomeric compounds, i.e. small molecules, and oligomeric compounds, i.e. oligo- and polypeptides, peptidomimetics, oligo- and polysaccharides, oligo- and polynucleotides,. Such compound(s) could be a potential inhibitor, an agonist, an antagonist, a transcription factor, a substrate for an enzyme, a catalyst, a toxin, a hormone, an antigen, an antibody or a fragment thereof, a polynucleotide, or a derivative thereof.

The library may be created by solid-phase combinatorial or parallel synthesis, for example a "split-and-mix" synthesis (figure 2). A large assembly of beads is suspended in a suitable solvent in a parent container. The beads are provided with a

photocleavable linker having a reactive site. The reactive site is protected by an optional protecting group. In a first step of the synthesis, the beads are divided for coupling into separate containers. The protecting groups are then removed and a first portion of the molecule to be synthesized is added to the various containers. For the purpose of this brief description, the number of containers will be limited to three and the chemical entities denoted as A, B, C, D, E, and F. The protecting groups are then removed and a first portion of the molecule to be synthesized, i.e., the first chemical entity, is added to each of the three containers (i.e., A is added to container 1, B is added to container 2 and C is added to container 3). Thereafter, the various beads are washed of excess reagents as appropriate, and remixed in a parent container. Again, it will be recognized that by virtue of the large number of beads utilized at the outset, there will similarly be a large number of beads randomly dispersed in the parent container, each functionalized with a particular first chemical entity. Thereafter, the various beads are again divided for coupling in another group of three containers. The beads in the first container are deprotected and exposed to a second chemical entity (D), while the beads in the second and third containers are coupled to chemical entities E and F respectively. Accordingly, molecules AD, BD, and CD will be present in the first container, while AE, BE, and CE will be present in the second container, and molecules AF, BF, and CF will be present in the third container. Each bead, however, will solely display a resulting single molecular entity. Thus, all possible compounds formed from the first portions A, B, C, and the second portions, D, E, F have been formed. The beads are then recombined into one container and additional steps such as are conducted to complete the synthesis of the combinatorial library.

In another aspect the invention concerns a kit for screening a one-bead-one-compound library of compounds comprising:

i. a plurality of solid supports/beads, each solid support/bead having immobilized therein a plurality of compounds in a "one-bead-one-compound" fashion,

ii. one or more aqueous media(s) for washing and/or containing said solid supports/beads,

iii. a physicochemical or biological system for interaction with said compounds, and

iv. a substrate for said physicochemical or biological system.

In another aspect, the kit for screening a one-bead-one-compound library of compounds comprising:

i. a plurality of beads, each bead having immobilized therein a plurality of compounds in a "one-bead-one-compound" fashion, the compounds being immobilized on the solid support through a photolytically and/or chemically cleavable and/or enzymatically cleavable linker,

ii. one or more aqueous media(s) for washing and/or containing said solid supports/beads,

iii. a physicochemical or biological system for interaction with said compounds, and

iv. a substrate for said physicochemical or biological system.

The kit may comprise two or more physicochemical or biological systems and corresponding substrates for testing the solid supports/beads in two or more assays for different interactions.

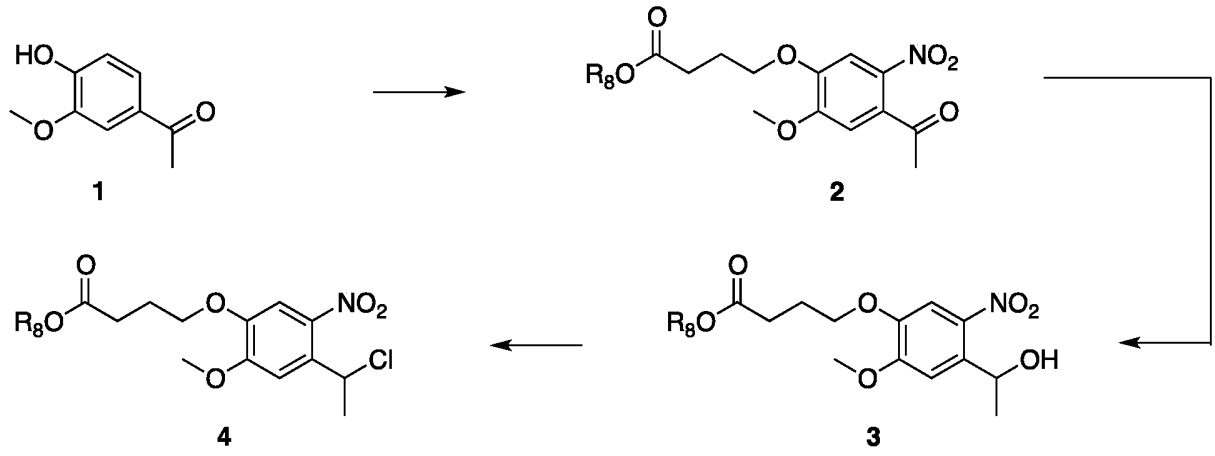
The kit according to the present invention is for screening a library wherein the immobilized compound(s) are selected from the group of a non-oligomeric compounds, i.e. small molecules, and oligomeric compounds, i.e. oligo- and polypeptides, peptidomimetics, oligo- and polysaccharides, oligo- and polynucleotides.

In one embodiment, the kit is for screening a library, wherein the compound(s) is/are a potential inhibitor, an agonist, an antagonist, a transcription factor, a substrate for an enzyme, a catalyst, a toxin, a hormone, an antigen, an antibody or a fragment thereof, a polynucleotide, or a derivative thereof.

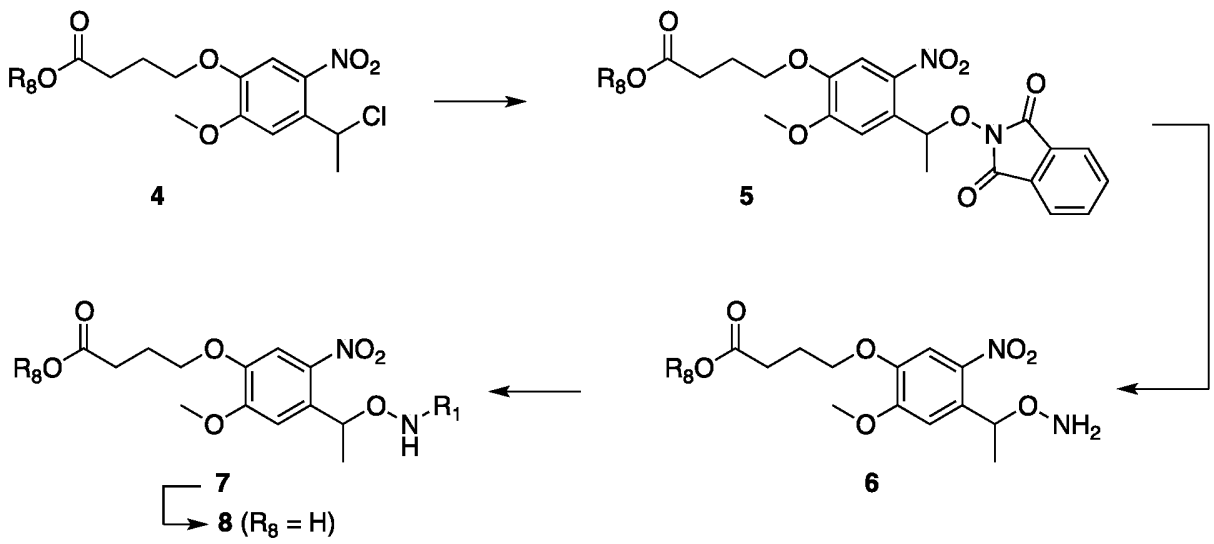
In another embodiment, the kit comprises a physicochemical or biological system selected from the group consisting of proteins, such as an enzyme, receptor, toxin, hormone, antibody or fragment thereof, antigen, polynucleotide, such as a gene or a regulator region, i.e. strands of DNA, or a genetic transcript, i.e. strands of RNA.

As an example, the screening of putatively active hydroxamic acids as HDAC inhibitors was demonstrated. Screening experiments were carried out using an HDAC Fluorimetric Assay/Drug Discovery Kit commercially available from Enzo Life Sciences.

The hydroxylamine linker 8 may serve as the starting point for the combinatorial synthesis of hydroxamic acid libraries. By way of example, the class of the compounds according to the present invention, may be prepared starting from acetovanilone 1 from which ketone 2 can be prepared in a few high-yielding steps. Reduction of the ketone to the corresponding alcohol, followed by chlorination with thionylchloride in CH_2Cl_2 affords the key intermediate chloride 4.



Substitution of chloride can be effected by reaction with *N*-hydroxyphthalimide to give 5. Treatment of 5 with hydrazine removes the phthalimido group to give 6, which can then be protected with a protecting group R_1 (e.g. Fmoc) to give the protected hydroxylamine-ester 7. Selective hydrolysis of the ester group R_8 may be accomplished by any suitable chemical or biological hydrolysis process, for example by use of an appropriate esterase, thus affording the R_1 (e.g. Fmoc)-protected hydroxylamine-functionalized carboxylic acid linker 8. Novozyme 435 is one example of a suitable esterase for a selective removal of the ester group R_8.



Using standard TBTU-mediated peptide coupling reactions, linker **6** was employed for the combinatorial synthesis of a library of putative HDAC inhibitors.

For the enzyme inhibition assay, differently functionalized library beads were placed in a flat-bottomed glass dish and a buffered aqueous solution added prior to irradiation with UV-light (360 nm) to release compounds into the interior of each bead. Afterwards an aqueous buffer solution of HDAC substrate was added and the mixture left to equilibrate for some time, which resulted in absorption or intake of substrate from the aqueous solution into the organic environment inside the beads. The surrounding substrate solution was removed, followed by washing the beads once with pure buffer. Finally, buffer followed by HeLa HDAC extract solution were added and allowed to equilibrate for a sufficient time. The HDAC reaction was developed by the addition of a developer solution containing the known inhibitor TSA to simultaneously quench any further deacetylation reaction. A blue coloration of beads upon inspection of the plates under a fluorescence microscope indicates that no inhibition of HDAC activity has taken place. In contrast, beads remaining colorless indicate that HDAC activity was inhibited by the compounds released inside these beads (see Figure 4 and 5 for graphical illustrations of the in-bead HDAC assay in the presence of an inhibitor and non-inhibitor, respectively.).

To facilitate post-screening MALDI-TOF MS sequencing of active beads, a 4-bromophenylalanine spacer was positioned between a Rink-linker functionalized

solid support and the photolabile linker unit (Figure 1). Orthogonal cleavage of the acid-labile Rink linker (see Figure 1) provides a cleavage product with sufficient mass to be out of range of low-mass noise and matrix ions typically seen in the MALDI-TOF MS analysis. Furthermore, the 4-bromophenylalanine spacer generates mass peaks with a characteristic bromine isotope pattern, so that the relevant peaks of the library products are readily identified by the presence of two peaks of equal intensity [M+Na]+ (for the ^{79}Br-capped fragments) and [M+2+Na]+ (for the corresponding ^{81}Br-capped fragments).

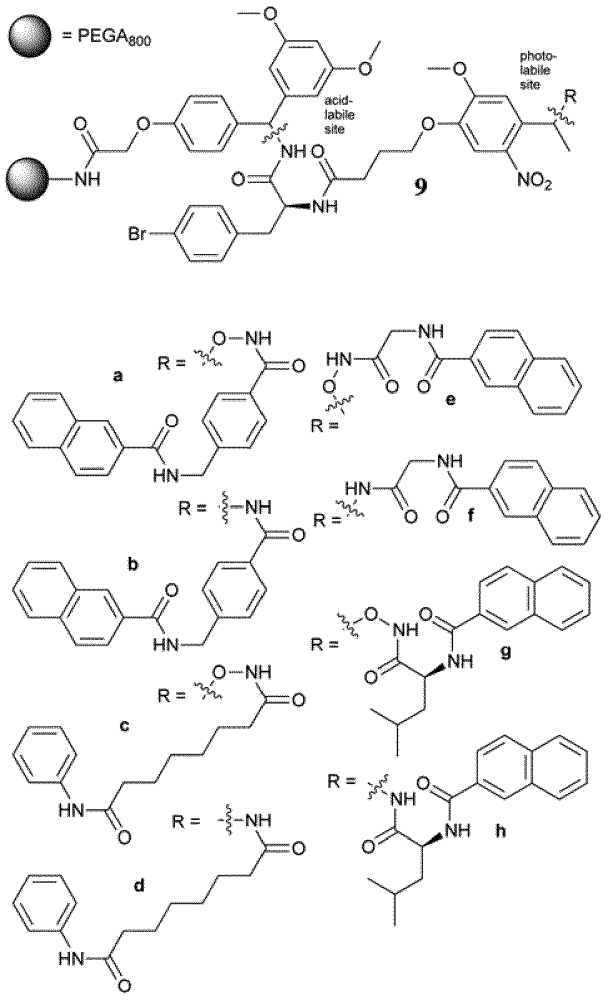
To show the possibility of controlling the concentration of substrate and inhibitor in the assay, two series of experiments were performed (see figure 6), keeping either the substrate concentration or the inhibitor concentration (photolysis time) constant; the constant factor acting as an internal standard. The results in figure 7 clearly illustrates that the concentration of substrate inside the beads are controlled by the concentration of substrate solutions added in the assay. Furthermore, figure 8 shows that the maximal amount of substrate that 3 mg of beads can take up is approximately 50 nmol, which amounts to substrate concentrations exceeding those normally used in biological assays.

In experiments with constant substrate concentration, it was shown that it is possible to control the concentration of inhibitor in the in-bead assay through a variation of photolysis time and/or the intensity of the light (see figure 9).

To further test the success of the "in-bead" technology according to the present invention, beads functionalized with SAHA (9c), an approved drug and known inhibitor of histone deacetylases, were tested in the assay. Inspection of the beads under a fluorescence microscope showed pronounced inhibition of HDAC activity in the presence of SAHA (Figure 10). Significantly, no active beads were detected when the photolysis step was excluded and immediately, a bright blue coloration of the beads was observed upon inspection of the plates under a fluorescence microscope. Furthermore, beads functionalized with a ligand without HDAC-inhibitory activity (9d) resulted in no inhibition of HDAC activity and accordingly no quenching of fluorescence was observed.

The colourless beads isolated from the assay were washed with aqueous buffer and CH_3CN to remove assay components before manually transferring one bead to a MALDI target. The active bead was swollen in TFA/CH_2Cl_2 on the MALDI target and left to react before being subjected to MALDI-TOF MS analysis, which showed the expected mass of the H_2N-(4Br)Phe-PLL-SAHA-fragment.

To validate the "in-bead" technology as a useful screening technique, a small bead-based library (9a-h), including beads functionalized with SAHA (9c), was screened. A photolysis time of 2 min produced approximately 20% beads of greater than 50% inhibition (Figure 11). Shortening the time to 0.5 min decreased the number of beads with greater than 50% inhibition to 10% (Figure 12). Evidently, the photolysis time affects the concentration of the inhibitor inside the bead. With this "in-bead" assay approach it is possible to control the concentration of ligand present in the assay, i.e. carry out dose-response experiments, and distinguish high- and low-affinity ligands.



The colorless beads detected in the assay were manually picked with a pipette and transferred to individual analysis tubes. Analysis of several of the colorless beads revealed that all of those beads contained SAHA (9c). Isolation and analysis of colored beads showed any of the other compounds 9a-b,d-h. These results demonstrate the value of this simple "in-bead" screening strategy for the identification of enzyme inhibitors.

Hydroxamic acids are strongly metal binding compounds and many metal-dependent biological systems may be probed with these compounds.

We envisioned the possibility of performing a dose-response assay by simply varying the light exposure time. Batches (3 mg) of two different library beads (figure 6) were illuminated for 5 s, 15 s, 1 min and 5 min, respectively, before being subjected to the in-bead HDAC assay (figure 13). As the fluorescence intensity may vary

slightly with the size of the bead and the size of the beads is not completely homogeneous, the fluorescence intensity were measured on each batch of exactly 3 mg of beads using a TECAN plate reader and plotted against light exposure time (figure 14 + 15). These results clearly demonstrate the possibility of performing dose-response measurements, which is a unique advantage offered by the in-bead technology compared with other bead-based techniques, which merely points to qualitative binding affinities.

The limited amount of compound used in an in-bead assay enables the performance of multiple assays with a single library bead. After thorough washing and drying of beads from one assay, the beads may be reused in another assay. Results of different biological screens can be directly compared, providing important information regarding the selectivity of compounds, aiding the prioritization of hits for the subsequent steps of hit-identification and re-synthesis. Applying library beads in a HDAC selectivity screen using the recombinant HDAC1 and 8 enzymes demonstrated this opportunity.

The "in-bead" screening technology provides a rapid, convenient, and efficient primary screening tool for bead-based combinatorial libraries. Regarding the ease of this method as a primary screening tool, the approach is relatively rapid in that a library can be screened in less than 1 h. Another significant advantage of this method is the low costs of the screening format that does not rely on costly robotics or automation instruments, and the screening operation, which can be carried out with small amounts of chemical and biological reagents. The screening results of the HDAC inhibitor library show that this screening method is capable of identifying high-affinity inhibitors from combinatorial bead-based libraries. Rapid and unambiguous sequencing of selected beads by MALDI-TOF MS may be facilitated by a combined acid- and photolabile cleavage construct. In addition to the identification of enzyme inhibitors, the described "in-bead" technology is a generally applicable method for evaluating other biological targets by adaptation to many other chemical or biological assay systems.

The present invention will be illustrated in the following non-limiting examples.

EXAMPLES

Example 1

Ethyl 4-(4-Acetyl-2-methoxyphenoxy)butanoate (1a).

To a solution of acetovanillone **1** (35.5 g, 0.21 mol; Sigma Aldrich, Denmark: W508454-1KG) in DMF (120 mL) was added K_2CO_3 (44.3 g, 0.32 mol) and ethyl 4-bromobutyrate (31.0 mL, 0.21 mol). The mixture was stirred for 16 hours at rt, then heated for 3 hours at 50 °C. After filtration the solution was partitioned between EtOAc and H_2O. The organic phase was washed several times with H_2O to remove the DMF. The organic phase was dried ($MgSO_4$), filtered and the solvent was removed by rotary evaporation to afford 58.9 g of **1a** (quant.) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.23 (t, *J* = 7.3 Hz, 3 H), 2.17 (pentet, *J* = 7.3 Hz, 2 H), 2.52 (t, *J* = 7.3 Hz, 2 H), 2.53 (s, 3 H), 3.88 (s, 3 H), 3.87-4.17 (m, 4 H), 6.87 (d, J = 8.4 Hz, 1 H), 7.47-7.53 (m, 2 H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.4, 24.5, 26.5, 30.8, 56.2, 60.7, 68.0, 110.6, 111.4, 123.4, 130.7, 149.5, 152.8, 173.3,196.7; UPLC/MS (ESI) *m/z* 281.3 $[MH]^+$.

Ethyl 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate (2).

A solution of ketoester **1a** (10.0 g, 35.7 mmol) in 30 mL acetic anhydride was slowly added to a solution of 70% HNO_3 (200 mL) and acetic anhydride (40 mL) at 0 °C. After stirring for 3 h the reaction mixture was poured into ice-cooled water. The precipitate was immediately collected by filtration (we found that leaving the mixture for longer time reduced the yield due to hydrolysis of the ester). The precipitate was washed extensively with water before being dried under vacuum to afford 10.8 g of **1b** (82%) as a pale yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.23 (t, *J* = 7.3 Hz, 3 H), 2,10 (pentet, *J* = 7.3 Hz, 2 H), 2.50 (t, J = 7.1 Hz, 2 H), 2.51 (s, 3 H), 3.83 (s, 3 H), 4.03 (t, *J* = 7.3 Hz, 2 H), 4.10 (q, J = 7.1 Hz, 2 H), 6.95 (s, 1 H), 7.57 (s, 1 H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.3, 24.3, 28.2, 30.4, 55.7, 59.6, 68.4, 108.6, 110.7, 132.8, 138.2, 148.8, 154.3, 173.2, 198.7; UPLC/MS (ESI) *m/z* 326.3 $[MH]^+$.

Ethyl 4-(4-(1-hydroxyethyl) -2-methoxy-5-nitrophenoxy)butanoate (3).

To a solution of **1b** (4.00 g, 12.3 mmol) in 300 mL MeOH at 0 °C was slowly added $NaBH_4$ (1.2 g; 31.7 mmol) in portions. After end addition the mixture was allowed to reach rt. The reaction was complete after 3 h (as judged by TLC). The reaction was quenched by addition of 200 mL of sat. NH_4Cl (aq). The reaction was extracted with EtOAc (400 mL), washed with water (2 × 300 mL) and saturated brine (300 mL). The organic phase was dried ($MgSO_4$), filtered and the solvent was removed by rotary evaporation to give 4.0 g of **2** (quant.) as a pale yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.20 (t, *J* = 7.2 Hz, 3 H), 1.48 (d, *J* = 7.0 Hz, 3 H), 2.10 (pentet, *J* = 7.0 Hz, 2 H), 2.47 (t, *J* = 7.1 Hz, 2 H), 3.90 (s, 3 H), 4.03 (t, *J* = 7.0 Hz, 2 H), 4.08 (q, *J* = 7.1 Hz, 2H), 5.48 (q, *J* = 7.0 Hz, 1 H), 7.23 (s, 1 H), 7.50 (s, 1 H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.9, 23.9, 27.8, 30.0, 56.0, 61.6, 68.1, 108.5, 109.8, 137.8, 138.5, 147.7, 153.8, 174.6; UPLC/MS (ESI) *m/z* 328.4 $[MH]^+$, 310.3 $[M - OH]^+$.

Ethyl 4-(4-(1-chloroethyl)-2-methoxy-5-nitrophenoxy)butanoate (4):
To a solution of **2** (4.0 g, 12.3 mmol) in 60 mL CH_2Cl_2 at 0 °C was added thionyl chloride (20 mL). The reaction was allowed to reach rt. The reaction was complete after 2 h (as judged by TLC). The reaction was evaporated to dryness and co-evaporated several times with toluene (3 × 20 mL). The residue was passed through a short plug of silica using EtOAc/heptane (1:1) as the eluent. Evaporation of the solvent gave 3.6 g of **3** (79%) as a pale yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.20 (t, J = 7.2 Hz, 3H), 1.80 (d, *J* = 7.1 Hz, 3 H), 2.10 (pentet, *J* = 7.0 Hz, 2 H), 2.47 (t, *J* = 7.1 Hz, 2 H), 3.92 (s, 3 H), 4.03-4.13 (m, 4 H), 5.86 (q, *J* = 7.1 Hz, 1 H), 7.23 (s, 1 H), 7.43 (s, 1 H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.5, 24.4, 27.6, 30.8, 54.0, 56.6, 60.8, 68.5, 108.7, 110.6, 133.0, 140.0, 147.8, 154.0, 173.1; UPLC/MS (ESI) *m/z* 346.4 $[MH]^+$, 310.3 $[M - Cl]^+$.

Ethyl 4-(4-(1-((1,3-dioxoisoindolin-2-yl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (5)
Ethyl 4-(4-(1-chloroethyl) -2-methoxy-5-nitrophenoxy)butanoate (**4**) (2.0 g, 5.7 mmol) and *N*-hydroxyphthalimide (1.1 g, 6.8 mmol) were dissolved in DMF (40 ml) and the reaction mixture was heated to 60 °C for 12 hours. Upon cooling to rt the

reaction was quenched by addition of water (100 ml). The mixture was extracted with EtOAc (200 ml) and the organic phase was washed with water (3 × 150 mL) and brine (150 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was passed through a short plug of silica using EtOAc/heptane (1:1) as the eluent to yield 2.6 g of **5** (95%) as a yellow solid.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.2 (t, *J* = 7.1 Hz, 3 H), 1.5 (d, *J* = 7.1 Hz, 3 H), 2.12 (pentet, *J* = 7.0 Hz, 2 H), 2.47 (t, *J* = 7.1 Hz, 2 H), 3.91 (s, 3 H), 4.05 (t, *J* = 7.0 Hz, 2 H), 4.10 (q, *J* = 7.0 Hz, 2 H), 4.99 (q, *J* = 7.0 Hz, 1 H), 7.20 (s, 1 H), 7.57 (s, 1 H), 7.88 (m, 4 H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.4, 22.4, 24.4, 30.8, 56.7, 68.5, 109.3, 109.4, 123.7, 132.0, 132.2, 132.4, 140.0, 147.6, 154.4, 161.0, 173.0; UPLC/MS (ESI) *m/z* 473.2 $[MH]^+$.

Ethyl 4-(4-(1-(aminooxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (6)

Ethyl 4-(4-(1-((1,3-dioxoisoindolin-2-yl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (**5**) (2.6 g, 5.5 mmol) was dissolved in EtOH (30 ml). Hydrazine monohydrate (0.5 mL, 11.0 mmol) were added and the reaction mixture was refluxed for 2 hours. The resulting white precipitate was removed by filtration and the filtrate was concentrated *in vacuo* to give 1.9 g of **6** (quant.) as a yellow oil.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.2 (t, *J* = 7.1 Hz, 3 H), 1.5 (d, *J* = 7.1 Hz, 3 H), 2.01 (bs, 2H), 2.14 (pentet, *J* = 7.0 Hz, 2 H), 2.45 (t, *J* = 7.1 Hz, 2 H), 3.91 (s, 3 H), 4.05 (t, *J* = 7.0 Hz, 2 H), 4.10 (q, *J* = 7.0 Hz, 2 H), 4.99 (q, *J* = 7.0 Hz, 1 H), 7.28 (s, 1 H), 7.62 (s, 1 H); UPLC/MS (ESI) *m/z* 343.3 $[MH]^+$.

Ethyl 4-(4-(1-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (7)

Ethyl 4-(4-(1-(aminooxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (**6**) (1.9 g, 5.5 mmol) was dissolved in dioxane (10mL) and 10% aq. Na_2CO_3 (20mL) was added. The reaction mixture was cooled to 0 °C. A solution of Fmoc-Cl (1.6g; 6.0 mmol) in dioxane (10 mL) was slowly added. The reaction mixture was allowed to reach rt and stirred for 2h. The resulting yellow precipitate was isolated by filtration and washed several times with water. The product was lyophilized to give 2.9 g of the Fmoc-protected hydroxylamine-ethylester **7** (92%) as a yellow solid.

^{1}H-NMR (300MHz, $CDCl_3$): δ = 1.3 (t, *J* = 7.1 Hz, 3 H), 1.5 (d, *J* = 7.1 Hz, 3 H), 2.15 (pentet, *J* = 7.0 Hz, 2 H), 2.48 (t, *J* = 7.1 Hz, 2 H), 3.93 (s, 3 H), 4.13 (t, *J* = 7.0 Hz, 2 H), 4.10 (q, *J* = 7.0 Hz, 2 H), 4.78 (q, *J* = 7.0 Hz, 1 H), 7.28 (s, 1 H), 7.33 (m, 4H), 7.55 (d, 2H), 7.62 (s, 1 H); 7.88 (d, 2H), 8.01 (bs, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.4, 22.4, 24.4, 30.8, 47.2, 56.7, 61.3, 67.3, 68.5, 75.5, 109.4, 120.5, 123.7, 124.2, 126.3, 126.8, 130.0, 143.4, 149.0, 153.1, 154.4, 155.2, 156.3, 173.0; UPLC/MS (ESI) *m/z* 565.2 $[MH]^+$.

4-(4-(1-(((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (8)

Ethyl 4-(4-(1-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (**7**) (2.9 g, 5.1 mmol) was dissolved in a mixture of di-oxane and $KHPO_4$-buffer (5:1, 50 mL) and Novozyme 435 (2 g) was added. The reaction mixture was shaken for 5 days and filtered over a plug of celite to give 2.7 g of Fmoc-protected hydroxylamine-functionalized carboxylic acid linker **8** (quant.) as a yellow solid.

^{1}H-NMR (300MHz, $CDCl_3$): δ = 1.5 (d, *J* = 7.1 Hz, 3 H), 2.15 (pentet, *J* = 7.0 Hz, 2 H), 2.48 (t, *J* = 7.1 Hz, 2 H), 3.93 (s, 3 H), 4.13 (t, *J* = 7.0 Hz, 2 H), 4.78 (q, *J* = 7.0 Hz, 1 H), 7.28 (s, 1 H), 7.33 (m, 4H), 7.55 (d, 2H), 7.62 (s, 1 H); 7.88 (d, 2H), 8.01 (bs, 1H), 10.97 (bs, 1H); UPLC/MS (ESI) *m/z* 537.4 $[MH]^+$.

Example 2

Solid-Phase Synthesis

Attachment of Fmoc-Rink linker to amino functionalized PEGA$_{800}$ beads

Fmoc-Rink linker (3 eq.), NEM (4 equiv.) and TBTU (2.88 equiv.) were mixed in DMF, and shaken for 5 min at rt. The solution was then added to amino-functionalized PEGA$_{800}$ beads pre-swelled in DMF and allowed to react for 2 hours, followed by washing with DMF (× 6). Full conversion was judged by conventional Kaiser test.

Removal of the Fmoc protecting group was accomplished with 20% piperidine in DMF for 5 min. After washing twice with DMF, the deprotection procedure was re-

peated with a reaction time of 30 min. The solid support was finally washed with DMF (× 8).

Attachment of Fmoc-4-bromophenylalanine to Rink linker-functionalized $PEGA_{800}$ beads

Fmoc-4-bromophenylalanine (3 equiv.) was dissolved in DMF, and NEM (4 equiv.) followed by TBTU (2.88 equiv.) were added. The mixture was shaken for 5 min at room temperature before being added to the Rink-functionalized beads pre-swelled in DMF. The mixture was shaken for 2 h at room temperature. The solid support was washed with DMF (× 6). Full conversion was judged by conventional Kaiser test.

Fmoc deprotection was accomplished as noted above before. The solid support was finally washed with DMF (× 8), MeOH (× 6), CH_2Cl_2 (× 6) before being lyophilized.

Attachment of 4-(4-(1-(((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid to 4-bromophenylalanine-Rink linker-functionalized $PEGA_{800}$ beads

4-(4-(1-(((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (**8**) (3 equiv.) was dissolved in DMF, and NEM (4 equiv.) followed by TBTU (2.88 equiv.) were added. The mixture was shaken for 5 min at room temperature before being added to (4-Br)Phe-Rink linker-functionalized $PEGA_{800}$ beads pre-swelled in DMF. The mixture was shaken for 2 h at room temperature. The solid support was washed with DMF (× 6), MeOH (× 6) and CH_2Cl_2 (× 6) before being lyophilized. Full conversion was judged by conventional Kaiser test.

CLAIMS

1. A method for screening a library of compounds for their interaction with a physicochemical or biological system, comprising:

    a) obtaining a one-bead-one-compound library wherein the compounds are immobilized on a solid support,

    b) adding a physicochemical or biological system to said library in an aqueous media,

    c) detecting solid support(s) in said library showing interaction between said compound(s) and the physicochemical or biological system, and

    d) identifying compound(s) immobilized in said solid support(s) showing said interaction.

2. A method for screening a library of compounds for their interaction with a physicochemical or biological system according to claim 1, comprising:

    a) obtaining a one-bead-one-compound library wherein the compounds are immobilized on a solid support through a photolytically cleavable linker and/or a chemically cleavable linker and/or an enzymatically cleavable linker,

    b) releasing said immobilized compounds inside said solid supports by photolytic, chemical or enzymatic cleavage in an aqueous media,

    c) adding a physicochemical or biological system to said library in an aqueous media,

    d) detecting solid supports(s) in said library showing interaction between said compound(s) and the physicochemical or biological system, and

    e) identifying compound(s) immobilized in said solid supports(s) showing said interaction.

3. A method according to claim 2, wherein

    i. step b is performed before step c, or

ii. step c is performed before step b.

4. A method according to any of the claims 1-3, wherein said identification involves:

i. isolating solid support(s) showing said interaction, and

ii. identifying the compound(s) in said isolated solid support(s).

5. A method according to claim 4, wherein said identification involves:

i. releasing immobilized compounds from said isolated solid support(s) by photolytic, chemical or enzymatic cleavage, and

ii. identifying said released compound(s).

6. A method according to any one of the claims 1-5, wherein said compounds are selected from the group of non-oligomeric compounds, preferably small molecules, and oligomeric compounds, preferably oligo- and polypeptides, peptidomimetics, oligo- and polysaccharides, oligo- and polynucleotides.

7. A method according to any one of the claims 1-6, wherein the library is created by solid-phase combinatorial or parallel synthesis.

8. A method according to any one of the claims 1-7, wherein said adding of a physicochemical or biological system to said library comprises:

i. adding a substrate for said physicochemical or biological system,

ii. allowing said substrate to be absorbed into said solid supports,

iii. washing the beads in an aqueous media,

iv. adding said physicochemical or biological system,

v. allowing said physicochemical or biological system to be absorbed into said solid supports, and

vi. washing the solid supports in an aqueous media.

9. A method according to claim 8, wherein

a) steps i-iii are performed before steps iv-vi, or

b) steps iv-vi are performed before steps i-iii.

10. A method according to any of the claims 1-9, wherein the compound(s) is/are a potential inhibitor, an agonist, an antagonist, a transcription factor, a substrate for an enzyme, a catalyst, a toxin, a hormone, an antigen, an antibody or a fragment thereof, a polynucleotide, or a derivative thereof.

11. A method according to any of the claims 1-10, wherein the physicochemical or biological system is selected from the group consisting of proteins, such as an enzyme, receptor, toxin, hormone, antibody or fragment thereof, antigen, polynucleotide, such as a gene or a regulator region, i.e. strands of DNA, or a genetic transcript, i.e. strands of RNA.

12. A kit for screening a one-bead-one-compound library of compounds comprising:

i. a plurality of solid supports, each solid support having immobilized therein a plurality of compounds in a "one-bead-one-compound" fashion,

ii. one or more aqueous media(s) for washing and/or containing said solid supports,

iii. a physicochemical or biological system for interaction with said compounds, and

iv. a substrate for said physicochemical or biological system.

13. A kit for screening a one-bead-one-compound library of compounds according to claim 12 comprising:

i. a plurality of solid supports, each solid support having immobilized therein a plurality of compounds in a "one-bead-one-compound" fashion, the compounds being immobilized on the solid support through a photolytically cleavable linker and/or chemically cleavable linker and or enzymatically cleavable linker,

ii. one or more aqueous media(s) for washing and/or containing said solid supports,

iii. a physicochemical or biological system for interaction with said compounds, and

iv. a substrate for said physicochemical or biological system.

14. A kit according to claim 12 or 13, wherein the library is selected from the group of libraries wherein the compound(s) are selected from the group of non-oligomeric compounds, preferably small molecules, and oligomeric compounds, preferably oligo- and polypeptides, peptidomimetics, oligo- and polysaccharides, oligo- and polynucleotides.

15. A kit according to any of the claims 12-14, wherein said physicochemical or biological system is selected from the group consisting of proteins, such as an enzyme, receptor, toxin, hormone, antibody or fragment thereof, antigen, polynucleotide, preferably a gene or a regulator region thereof, single strands of DNA, a genetic transcript and RNA.

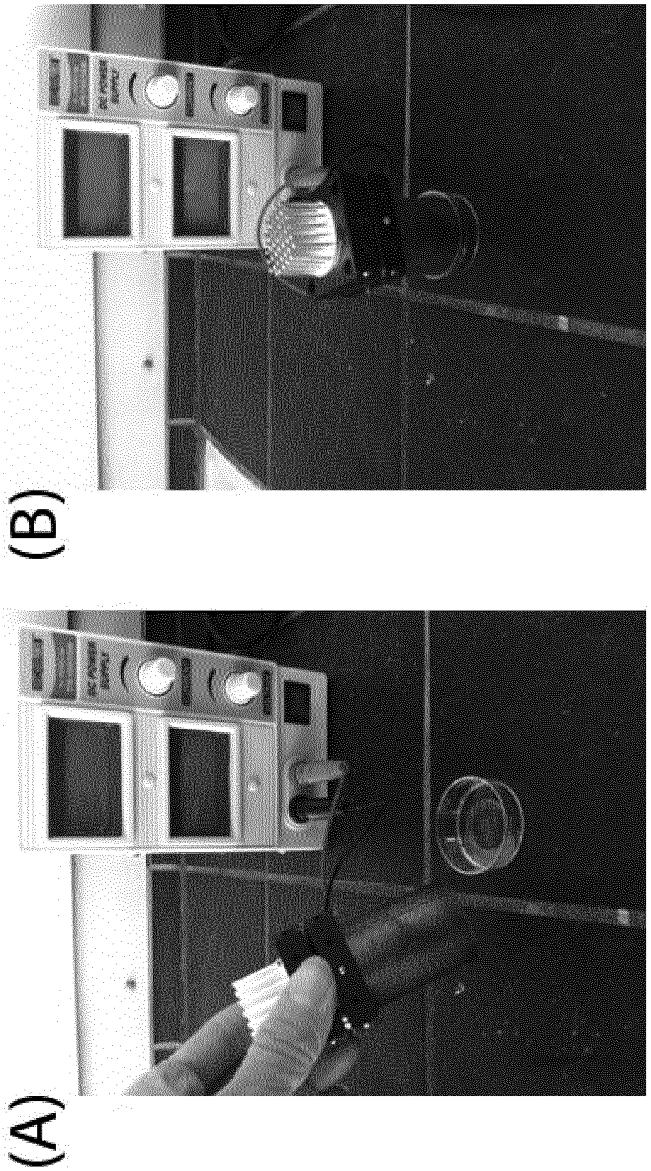


Figure 1

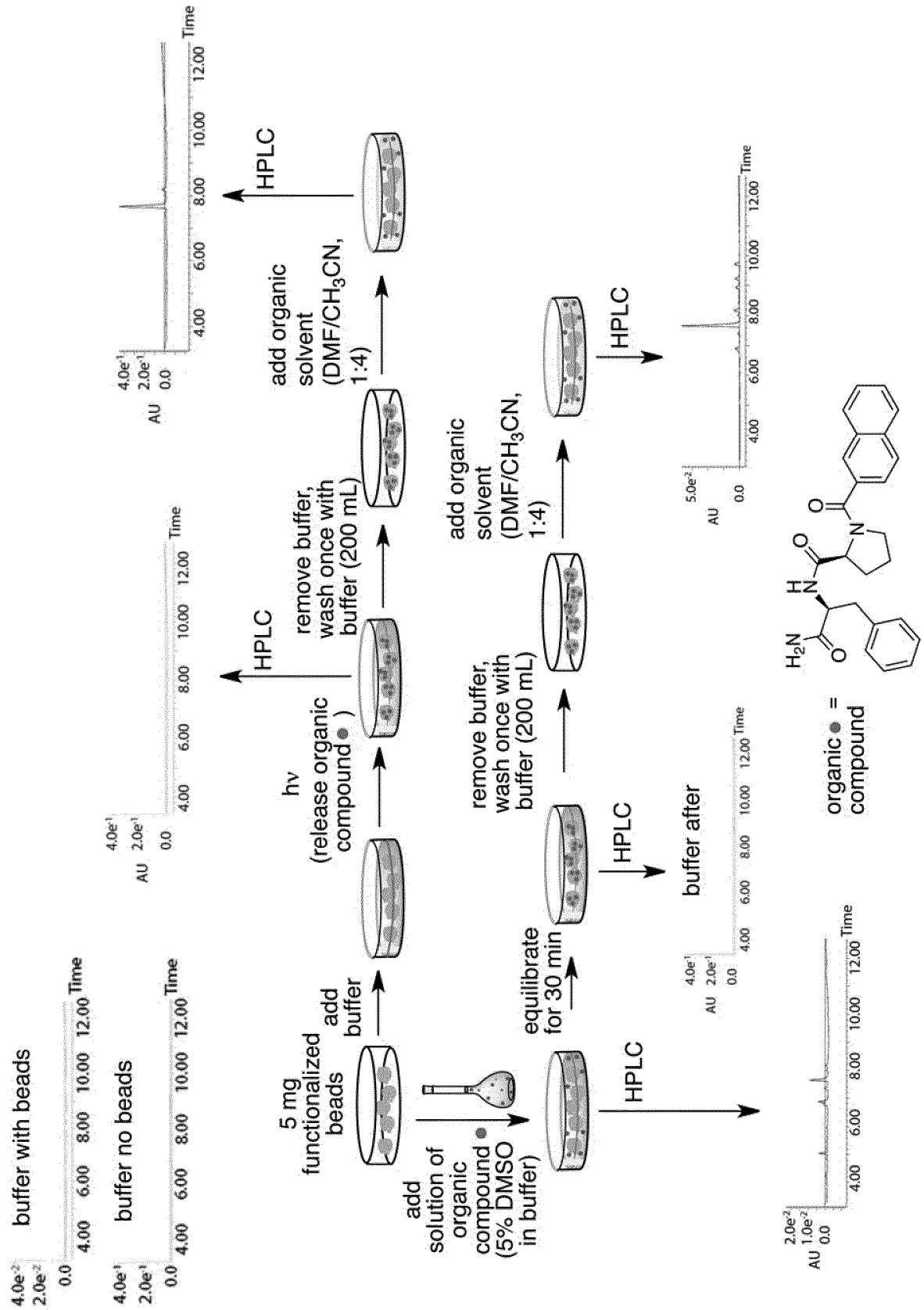

buffer with beads
buffer no beads
AU
Time
5 mg functionalized beads
add buffer
add solution of organic compound ● (5% DMSO in buffer)
HPLC
equilibrate for 30 min
hν (release organic compound ●)
remove buffer, wash once with buffer (200 mL)
add organic solvent (DMF/CH3CN, 1:4)
buffer after
organic ● = compound

Figure 2

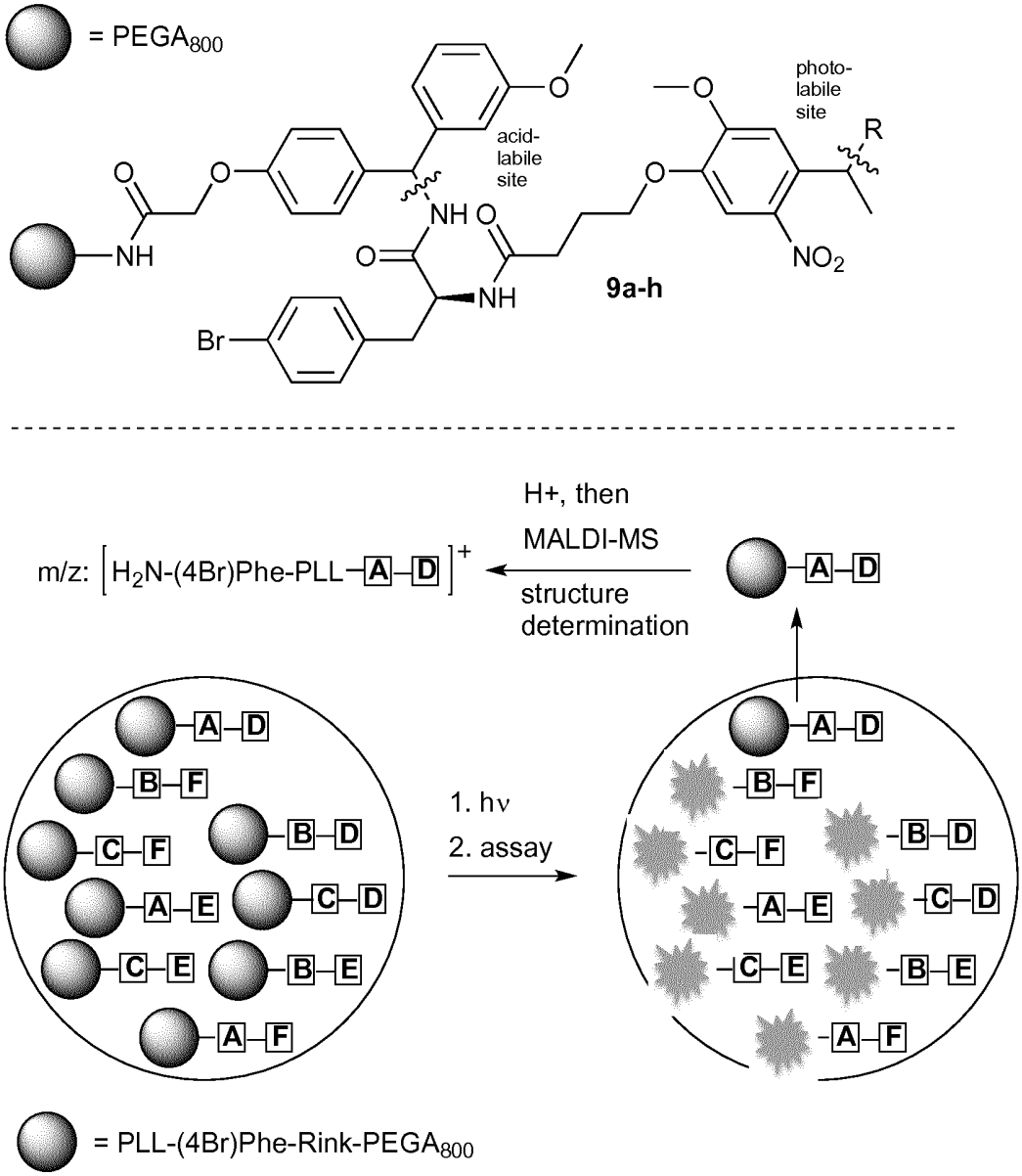

= PEGA800
photo-
labile
site
acid-
labile
site
R
NH
NO2
9a-h
Br
H+, then
MALDI-MS
structure
determination
m/z: [H2N-(4Br)Phe-PLL-A-D]+
A-D
B-F
C-F
B-D
A-E
C-D
C-E
B-E
A-F
1. hν
2. assay
= PLL-(4Br)Phe-Rink-PEGA800

Figure 3

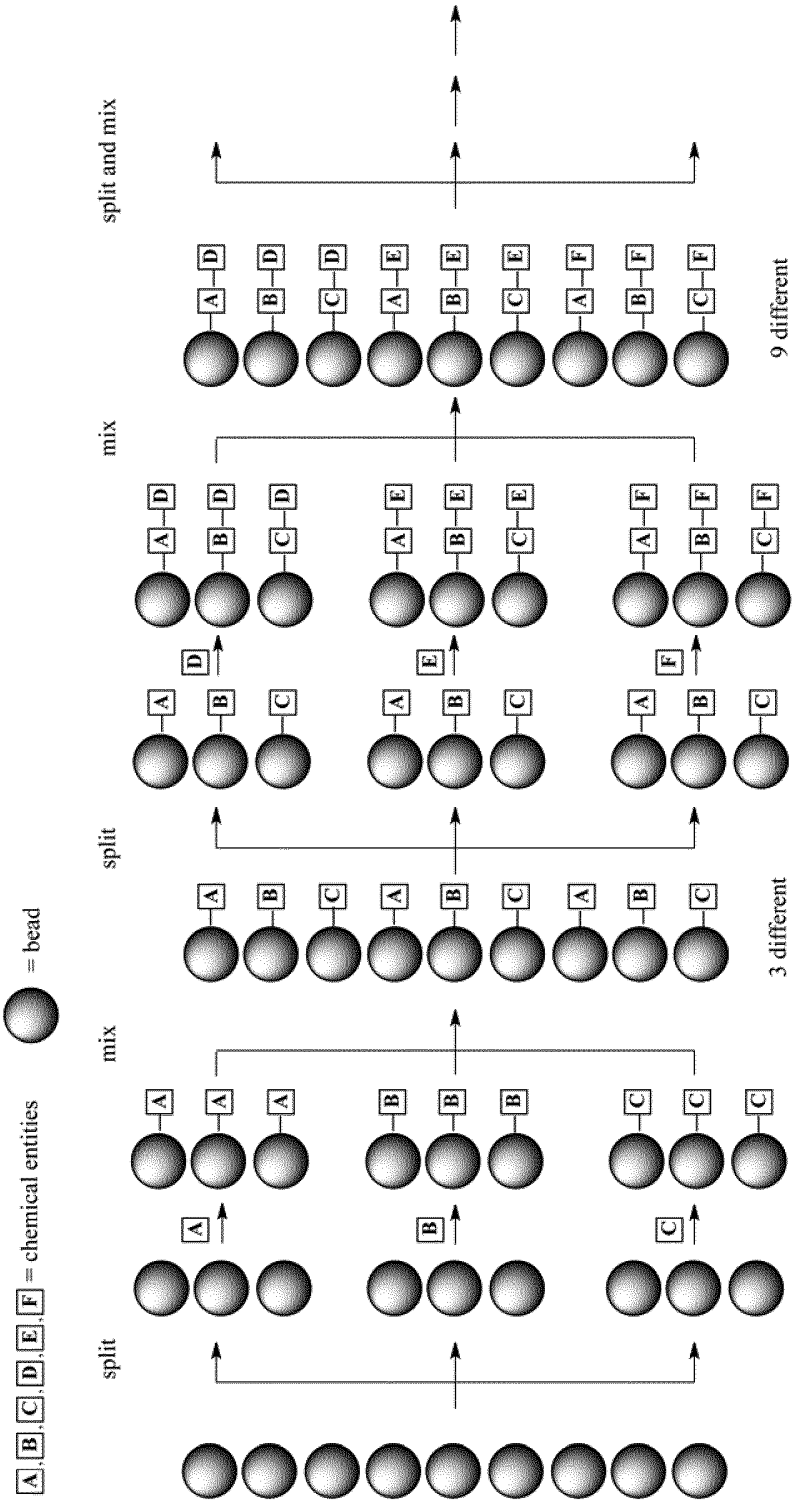

split
mix
split
mix
split and mix
3 different
9 different
= bead
A, B, C, D, E, F = chemical entities

Figure 4

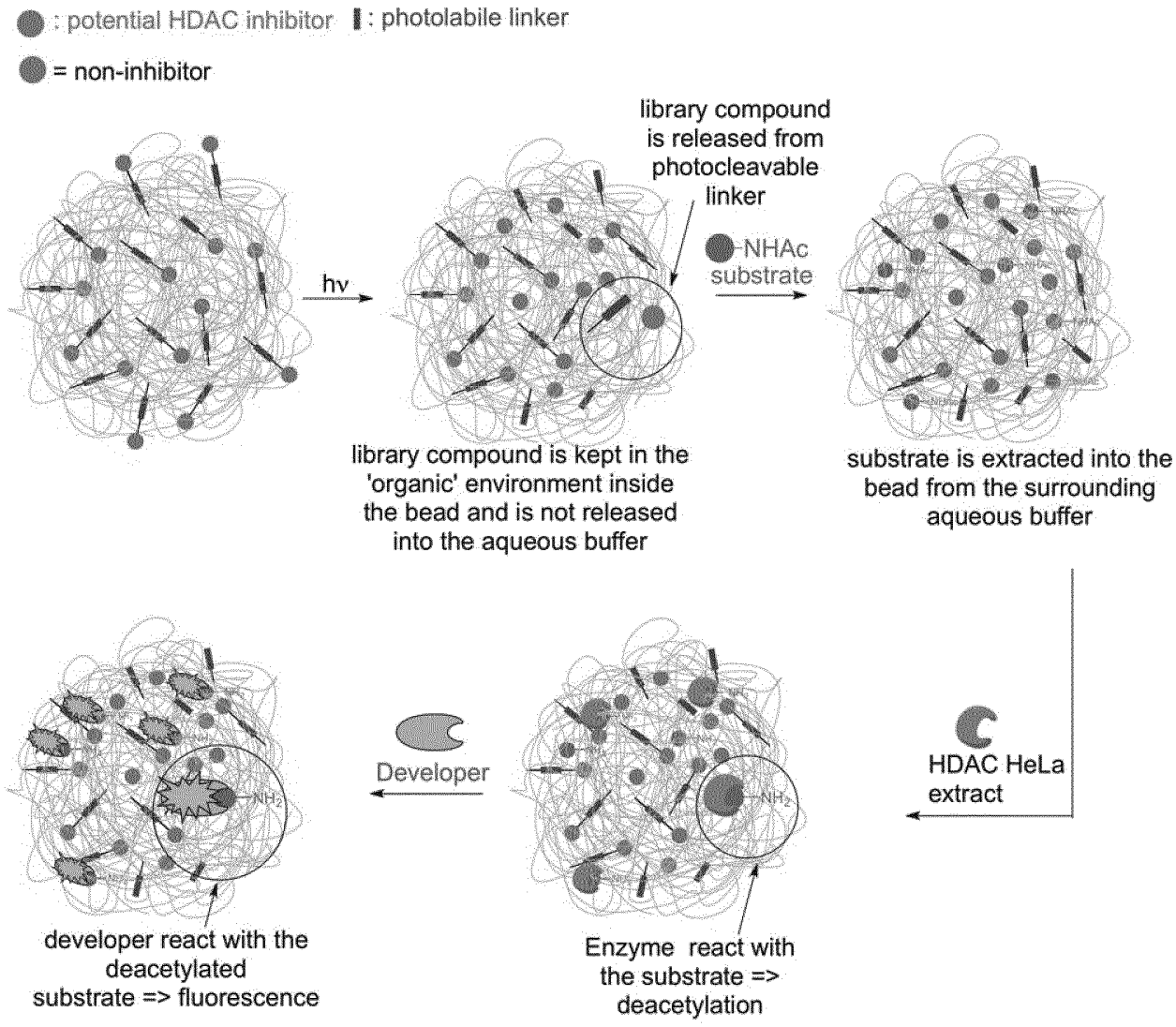

: potential HDAC inhibitor
: photolabile linker
= non-inhibitor
library compound is released from photocleavable linker
hν
NHAc substrate
library compound is kept in the 'organic' environment inside the bead and is not released into the aqueous buffer
substrate is extracted into the bead from the surrounding aqueous buffer
HDAC HeLa extract
Developer
NH2
developer react with the deacetylated substrate => fluorescence
Enzyme react with the substrate => deacetylation

Figure 5

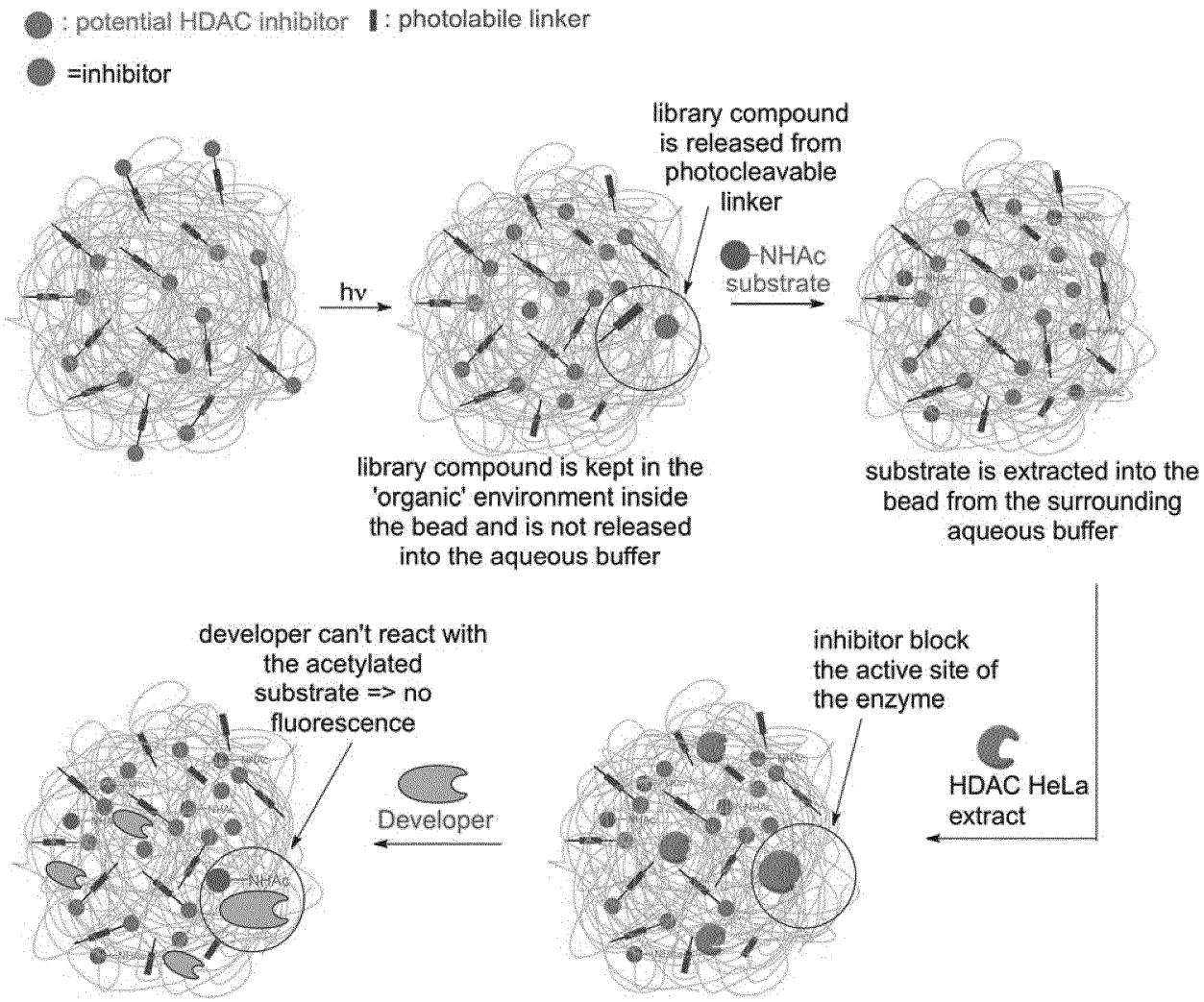

: potential HDAC inhibitor
: photolabile linker
=inhibitor
library compound is released from photocleavable linker
hν
NHAc substrate
library compound is kept in the 'organic' environment inside the bead and is not released into the aqueous buffer
substrate is extracted into the bead from the surrounding aqueous buffer
developer can't react with the acetylated substrate => no fluorescence
Developer
inhibitor block the active site of the enzyme
HDAC HeLa extract

Figure 6

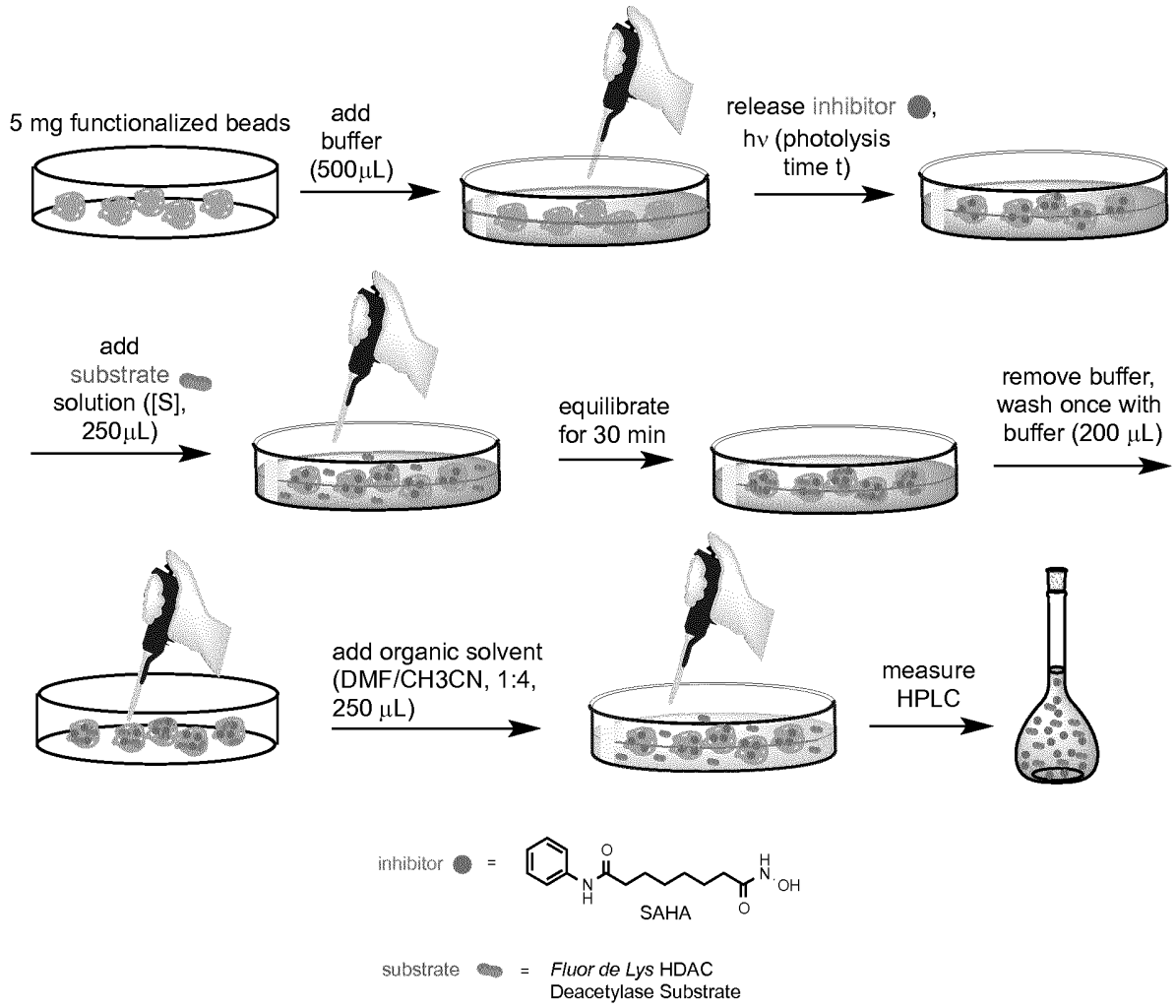

5 mg functionalized beads
add buffer (500μL)
release inhibitor ●, hν (photolysis time t)
add substrate ● solution ([S], 250μL)
equilibrate for 30 min
remove buffer, wash once with buffer (200 μL)
add organic solvent (DMF/CH3CN, 1:4, 250 μL)
measure HPLC
inhibitor ● =
SAHA
substrate ● = Fluor de Lys HDAC Deacetylase Substrate

Figure 7

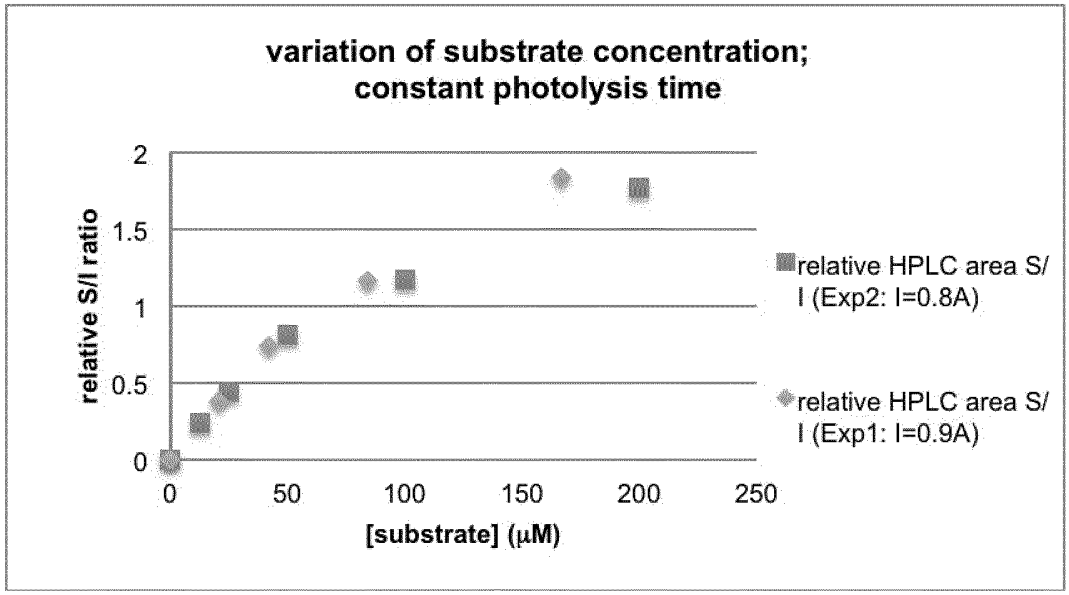

variation of substrate concentration; constant photolysis time
relative S/I ratio
2
1.5
1
0.5
0
0
50
100
150
200
250
[substrate] (μM)
relative HPLC area S/I (Exp2: I=0.8A)
relative HPLC area S/I (Exp1: I=0.9A)

Figure 8

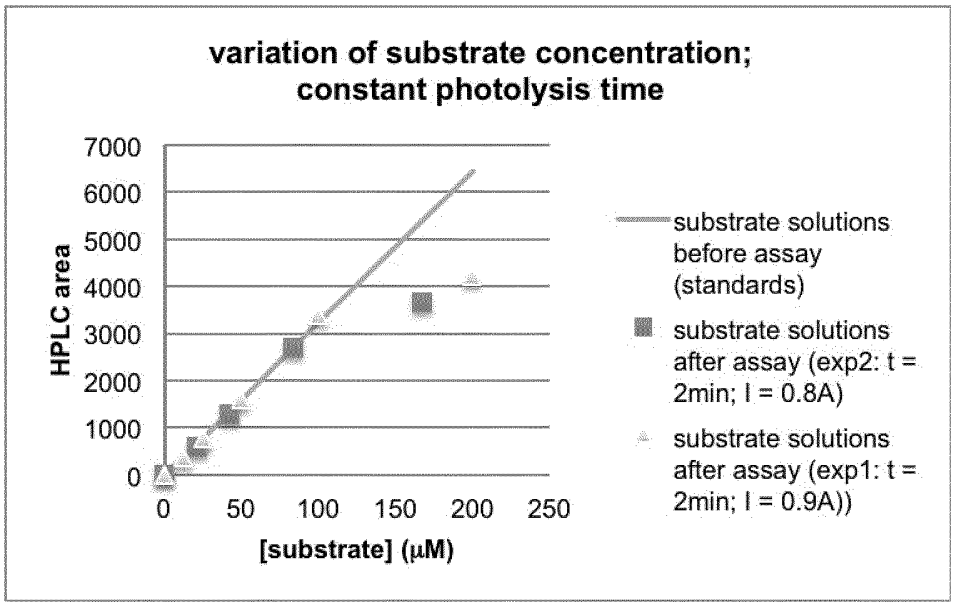

variation of substrate concentration; constant photolysis time
HPLC area
7000
6000
5000
4000
3000
2000
1000
0
0
50
100
150
200
250
[substrate] (μM)
substrate solutions before assay (standards)
substrate solutions after assay (exp2: t = 2min; I = 0.8A)
substrate solutions after assay (exp1: t = 2min; I = 0.9A))

Figure 9

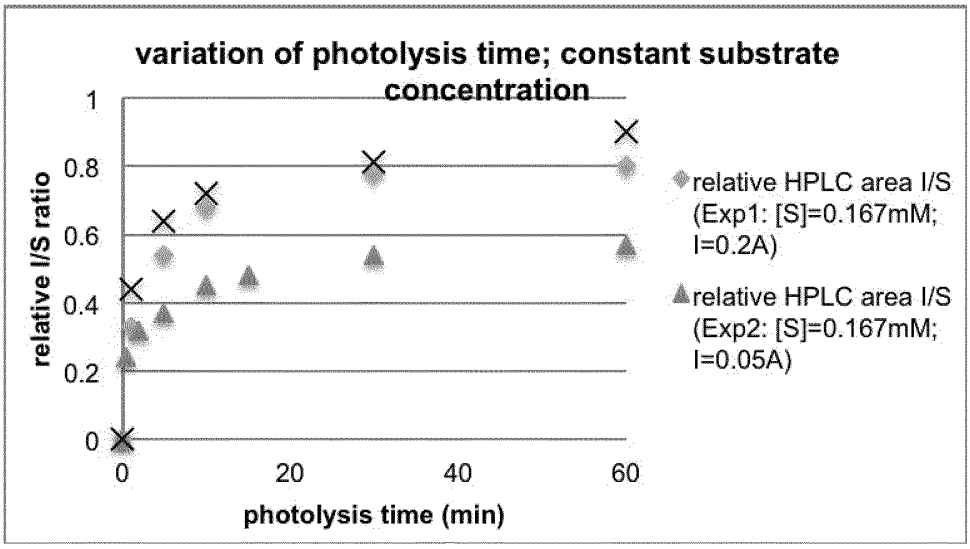

variation of photolysis time; constant substrate concentration
relative I/S ratio
1
0.8
0.6
0.4
0.2
0
0
20
40
60
photolysis time (min)
relative HPLC area I/S (Exp1: [S]=0.167mM; I=0.2A)
relative HPLC area I/S (Exp2: [S]=0.167mM; I=0.05A)

Figure 10

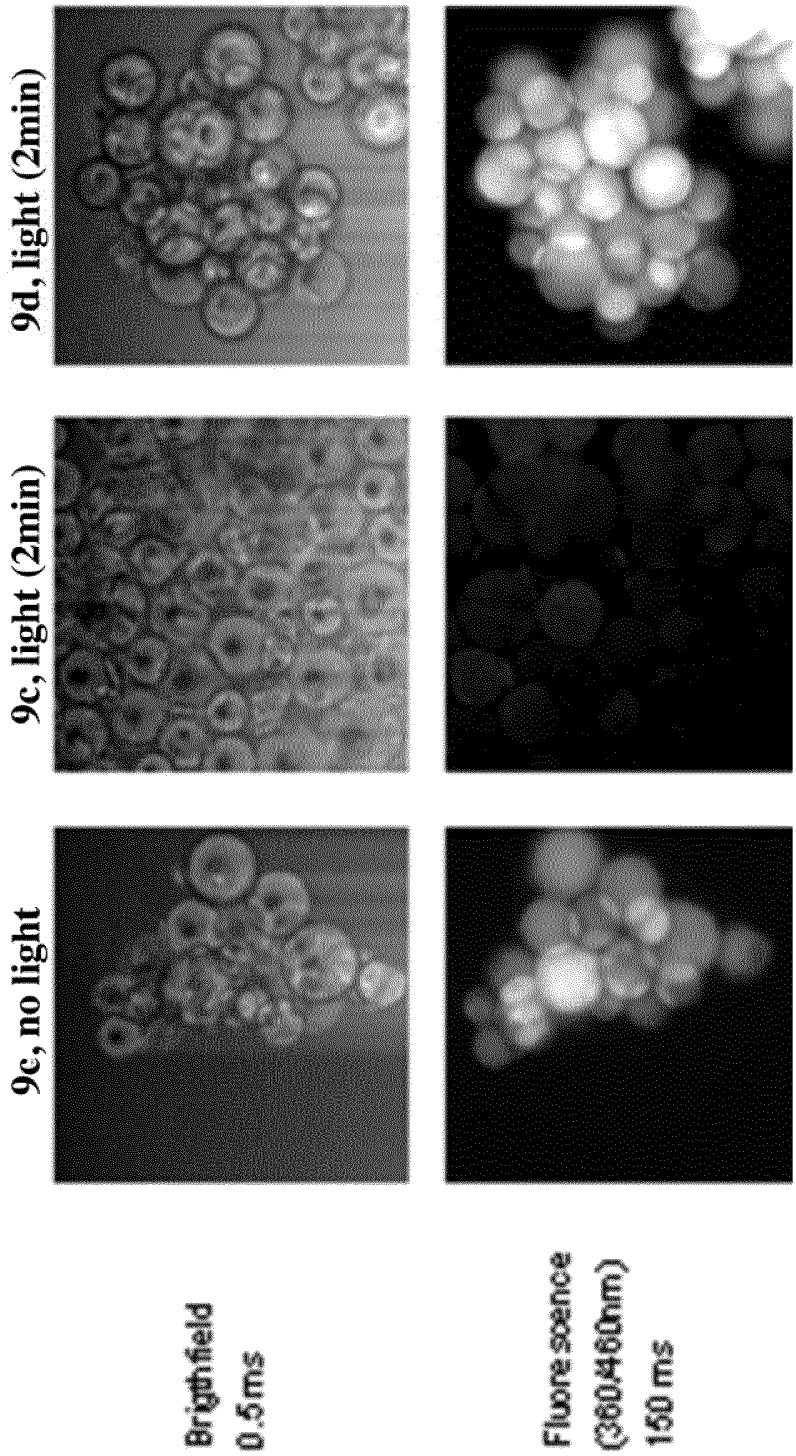

9d, light (2min)
9c, light (2min)
9c, no light
Brightfield
0.5ms
Fluorescence
(360,460nm)
150 ms

Figure 11

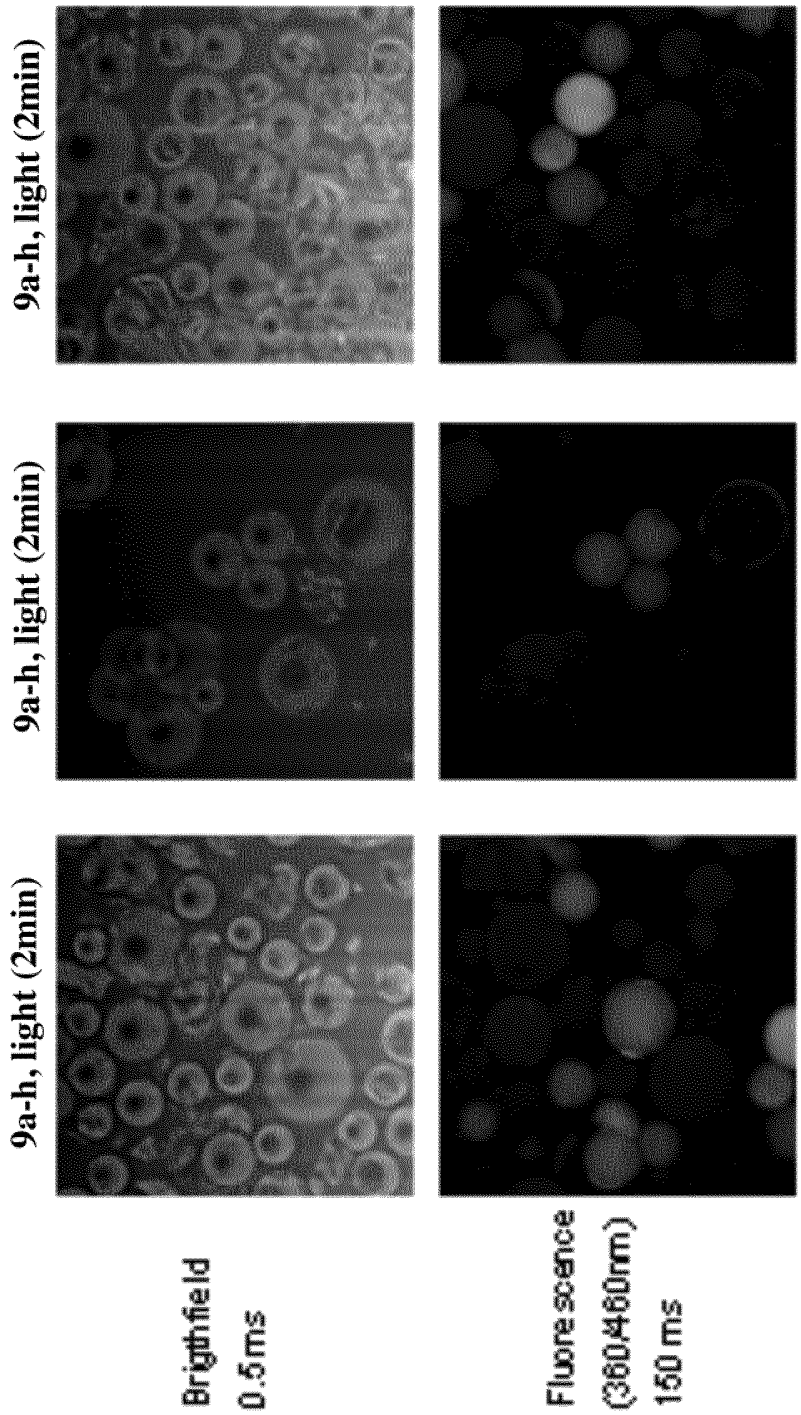

9a-h, light (2min)
9a-h, light (2min)
9a-h, light (2min)
Brightfield
0.5ms
Fluorescence
(360,460nm)
150 ms

Figure 12

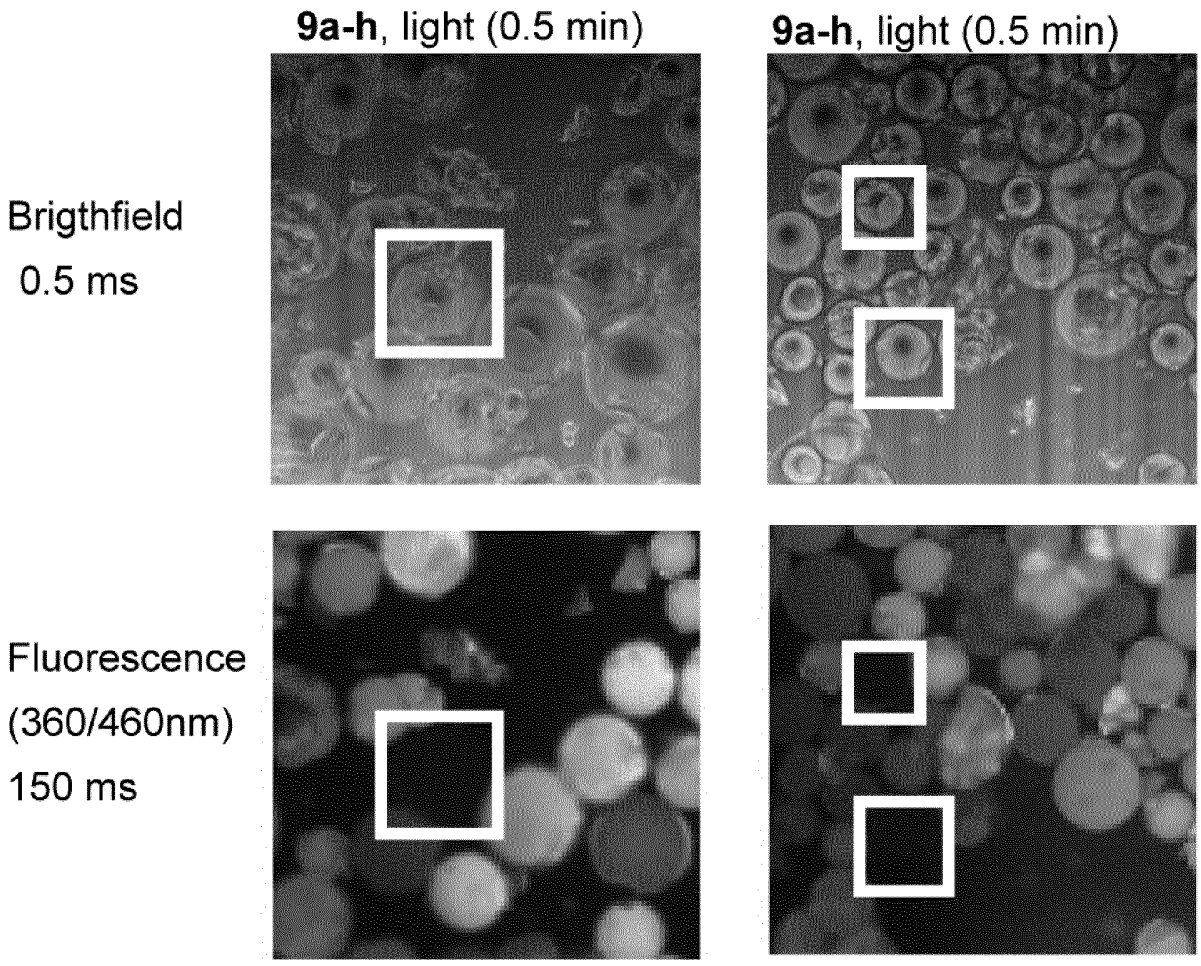
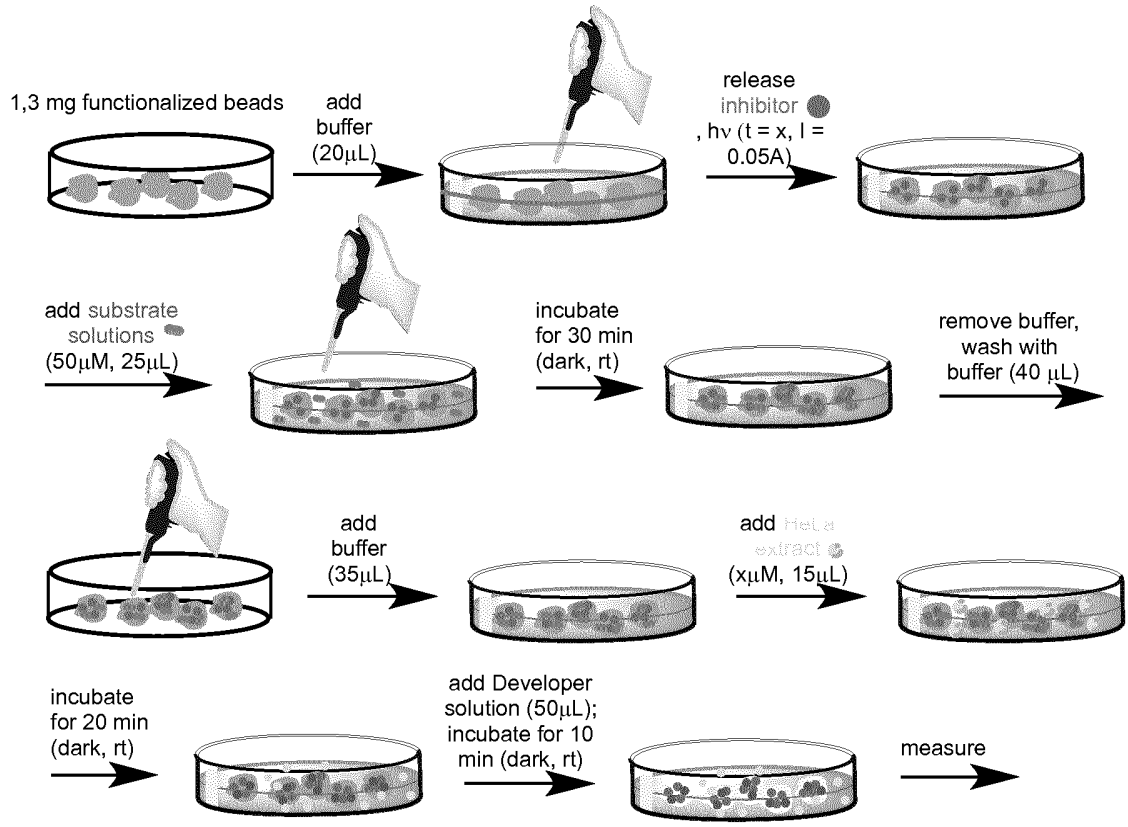
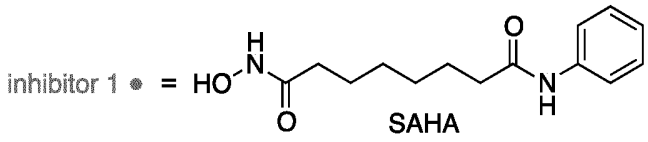
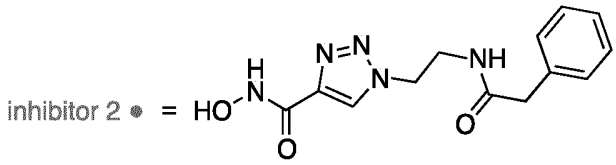

9a-h, light (0.5 min)
9a-h, light (0.5 min)
Brigthfield
0.5 ms
Fluorescence
(360/460nm)
150 ms

Figure 13

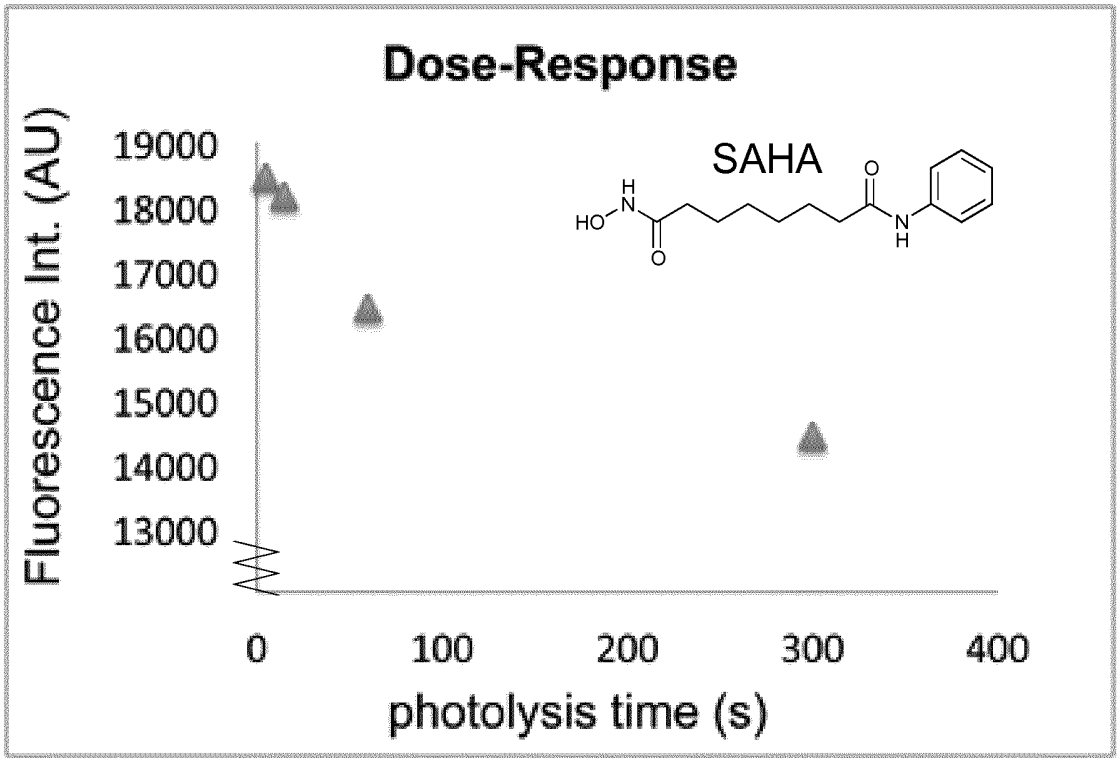






substrate = *Fluor de Lys* HDAC Deacetylase Substrate

Figure 14


Dose-Response
Fluorescence Int. (AU)
19000
18000
17000
16000
15000
14000
13000
SAHA
0
100
200
300
400
photolysis time (s)

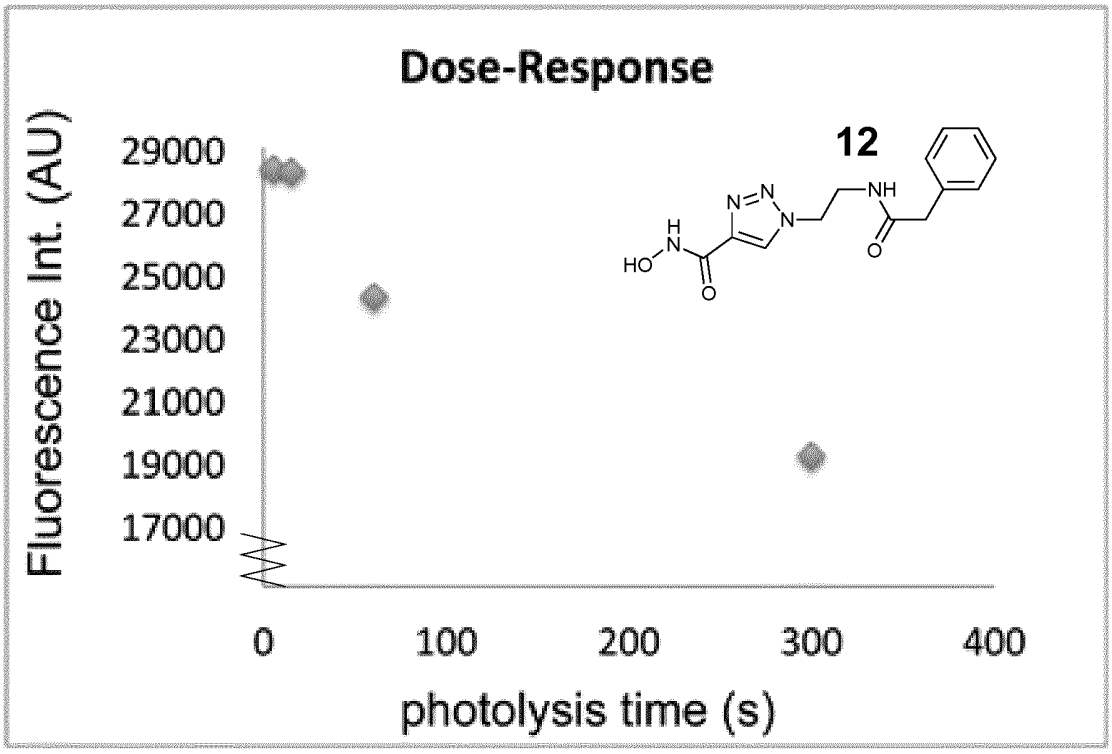

Dose-Response
Fluorescence Int. (AU)
29000
27000
25000
23000
21000
19000
17000
12
0
100
200
300
400
photolysis time (s)

Figure 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/070650

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2008/140230 A1 (SEOUL NAT UNIV IND FOUNDATION [KR]; LEE YOON-SIK [KR]; KIM BYUNG-GEE []) 20 November 2008 (2008-11-20) Claim 1; p. 14, 16; abstract -----	1-8, 10-15 9
A	X. WANG ET AL: "Applications of topologically segregated bilayer beads in 'one-bead one-compound' combinatorial libraries", JOURNAL OF PEPTIDE RESEARCH, vol. 65, no. 1, 1 January 2005 (2005-01-01), pages 130-138, XP055026406, ISSN: 1397-002X, DOI: 10.1111/j.1399-3011.2005.00192.x abstract ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 January 2013	30/01/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hohwy, Morten

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/070650

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